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# Cardiac cell fate control by the imidazoline $I_1$ receptor/nischarin: Application in cardiac pathology

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# Cardiac cell fate control by the imidazoline $I_1$ receptor/nischarin: Application in cardiac pathology

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# Résumé

La moxonidine, un médicament antihypertenseur sympatholytique de type imidazolinique, agit au niveau de la médulla du tronc cérébral pour diminuer la pression artérielle, suite à l'activation sélective du récepteur aux imidazolines $I_1$ (récepteur $I_1$, aussi nommé nischarine). Traitement avec de la moxonidine prévient le développement de l'hypertrophie du ventricule gauche chez des rats hypertendus (SHR), associé à une diminution de la synthèse et une élévation transitoire de la fragmentation d'ADN, des effets antiprolifératifs et apoptotiques. Ces effets se présentent probablement chez les fibroblastes, car l'apoptose des cardiomyocytes pourrait détériorer la fonction cardiaque. Ces effets apparaissent aussi avec des doses non hypotensives de moxonidine, suggérant l'existence d'effets cardiaques directes. Le récepteur $I_1$ se trouvé aussi dans les tissus cardiaques; son activation *ex vivo* par la moxonidine stimule la libération de l'ANP, ce qui montre que les récepteurs $I_1$ cardiaques sont fonctionnels malgré l'absence de stimulation centrale.

Sur la base de ces informations, en plus du i) rôle des peptides natriurétiques comme inhibiteurs de l'apoptose cardiaque et ii) des études qui lient le récepteur $I_1$ avec la maintenance de la matrix extracellulaire, on propose que, **à part les effets sympatholytiques centrales, les récepteurs $I_1$ cardiaques peuvent contrôler la croissance-mort cellulaire. L'activation du récepteur $I_1$ peut retarder la progression des cardiopathies vers la défaillance cardiaque, en inhibant des signaux mal adaptatifs de prolifération et apoptose.**

Des études ont été effectuées pour :

1. Explorer les effets *in vivo* sur la structure et la fonction cardiaque suite au traitement avec moxonidine chez le SHR et le hamster cardiomyopathique.
2. Définir les voies de signalisation impliquées dans les changements secondaires au traitement avec moxonidine, spécifiquement sur les marqueurs inflammatoires et les voies de signalisation régulant la croissance et la survie cellulaire (MAPK et Akt).
3. Explorer les effets *in vitro* de la surexpression et l'activation du récepteur $I_1$ sur la survie cellulaire dans des cellules HEK293.

4. Rechercher la localisation, régulation et implication dans la croissance-mort cellulaire du récepteur $I_1$ *in vitro* (cardiomyocytes et fibroblastes), en réponse aux stimuli associés au remodelage cardiaque : norépinephrine, cytokines (IL-1β, TNF-α) et oxydants ($H_2O_2$).

Nos études démontrent que la moxonidine, en doses hypotensives et non-hypotensives, améliore la structure et la performance cardiaque chez le SHR par des mécanismes impliquant l'inhibition des cytokines et des voies de signalisation p38 MAPK et Akt. Chez le hamster cardiomyopathique, la moxonidine améliore la fonction cardiaque, module la réponse inflammatoire/anti-inflammatoire et atténue la mort cellulaire et la fibrose cardiaque. Les cellules HEK293 surexprimant la nischarine survivent et prolifèrent plus en réponse à la moxonidine; cet effet est associé à l'inhibition des voies ERK, JNK et p38 MAPK. La surexpression de la nischarine protège aussi de la mort cellulaire induite par le TNF-α, l'IL-1β et le $H_2O_2$.

En outre, le récepteur $I_1$ s'exprime dans les cardiomyocytes et fibroblastes, son activation inhibe la mort des cardiomyocytes et la prolifération des fibroblastes induite par la norépinephrine, par des effets différentiels sur les MAPK et l'Akt.

Dans des conditions inflammatoires, la moxonidine/récepteur aux imidazolines $I_1$ protège les cardiomyocytes et facilite l'élimination des myofibroblastes par des effets contraires sur JNK, p38 MAPK et iNOS.

Ces études démontrent le potentiel du récepteur $I_1$/nischarine comme cible anti-hypertrophique et anti-fibrose à niveau cardiaque. L'identification des mécanismes cardioprotecteurs de la nischarine peut amener au développement des traitements basés sur la surexpression de la nischarine chez des patients avec hypertrophie ventriculaire. Finalement, même si l'effet antihypertenseur des agonistes du récepteur $I_1$ centraux est salutaire, le développement de nouveaux agonistes cardiosélectifs du récepteur $I_1$ pourrait donner des bénéfices additionnels chez des patients non hypertendus.

**Mots-clés** : Récepteur aux imidazolines $I_1$, moxonidine, hypertension, hypertrophie du ventricule gauche, défaillance cardiaque, cardiomyocyte, fibroblaste, HEK293, norépinephrine, cytokines.

# Abstract

Moxonidine, an antihypertensive sympatholytic imidazoline compound, reduces blood pressure by selective activation of non-adrenergic imidazoline $I_1$-receptors (also known as nischarin) in brainstem medulla. Moxonidine prevents left ventricular hypertrophy development in hypertensive rats, associated with reduced cardiac DNA synthesis and early transient increase in DNA fragmentation. It is likely that the anti-proliferative and apoptotic effects occur in fibroblasts, as cardiomyocyte apoptosis may deteriorate cardiac function. The effects also occurred to sub-hypotensive doses, suggesting a blood-pressure-independent mechanism and pointing to a local cardiac action. Imidazoline $I_1$-receptors have been identified in cardiac tissues, and their *ex vivo* activation by moxonidine stimulates ANP release, demonstrating that cardiac imidazoline $I_1$-receptors are functional without the contribution of the central nervous system.

Based on the above studies and on i) the role of natriuretic peptides in inhibition of myocardial cell apoptosis and ii) studies linking imidazoline $I_1$-receptors to the maintenance of the extracellular matrix and PC12 cell survival, we propose that **apart from centrally-mediated sympatholytic function, imidazoline $I_1$-receptors in the heart may control cell growth and death. Activation of imidazoline receptors may delay the progression of cardiac pathologies into heart failure by inhibition of maladaptive proliferative signalling and downstream apoptotic pathways.**

In order to test this hypothesis studies were performed to:

1. Explore the *in vivo* effects of moxonidine on cardiac structure and function in SHR and cardiomyopathic hamsters.
2. Define the pathways involved in the observed changes following moxonidine treatment, specifically, on inflammatory markers and pathways involved in LVH and cardiac cell survival/death (MAPK and Akt).
3. Explore *in vitro* the effect of imidazoline $I_1$-receptor activation by moxonidine, on cell survival by over-expressing nischarin in HEK293 cells, to circumvent the lack of specific imidazoline $I_1$-receptor agonists and antagonists.

4. Investigate *in vitro,* imidazoline $I_1$-receptor localization (cardiomyocytes and fibroblasts), regulation and implication in cell growth/death in response to cardiac remodelling-associated stimuli: norepinephrine, cytokines (IL-1β, TNF-α), and oxidants ($H_2O_2$).

The studies reveal that hypotensive and sub-hypotensive concentrations of moxonidine improve cardiac structure and performance in SHR by mechanisms that involve inhibition of cytokines, p38MAPK, and Akt signalling pathways. In cardiomyopathic hamsters moxonidine improves cardiac performance, in association with differential inflammatory/anti-inflammatory responses that culminate in attenuated cardiomyocyte death and fibrosis and altered collagen type expression. HEK293 cells, transfected with nischarin cDNA, show increased viability/proliferation in response to moxonidine. The overall survival response is associated with moxonidine's inhibition of ERK, JNK, and p38MAPK. Nischarin also opposes the reduced cell viability in response to oxidative stimuli (TNF-α, IL-1β and $H_2O_2$), with differential responses to moxonidine. Furthermore, the imidazoline $I_1$-receptor is expressed in cardiac fibroblasts and myocytes and its activation inhibits norepinephrine-induced cardiomyocyte death and fibroblast proliferation, through differential effects on MAPKs and Akt. Moxonidine/imidazoline $I_1$-receptor protects cardiomyocytes and facilitates elimination of myofibroblasts in inflammatory conditions, through opposite effects on JNK, p38MAPK and iNOS activity.

These studies emphasize the potential importance of imidazoline $I_1$-receptor/nischarin as an anti-hypertrophic and anti-fibrotic target. Identification of the cardio-protective mechanisms of cardiac nischarin could result in specifically-tailored cell/gene-driven nischarin treatments, which could be important for patients with heart disease. Also, while the antihypertensive action of centrally acting compounds is appreciated, new cardiac-selective $I_1$-receptor agonists may confer additional benefit.

**Keywords:** Imidazoline $I_1$-receptor, moxonidine, hypertension, left ventricular hypertrophy, heart failure, cardiomyocyte, fibroblast, HEK293, norepinephrine, cytokines.

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# List of abbreviations

ACE: Angiotensin converting enzyme

ANP: Atrial natriuretic peptide

ARB: Angiotensin receptor blocker

ATP: Adenosine triphosphate

BNP: Brain natriuretic peptide

CaMKII: Calcium/calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

CNP: C-type natriuretic peptide

CVD: Cardiovascular disease

DAG: Diacylglycerol

ECM: Extracellular matrix

EDV: End diastolic volume

eNOS: Endothelial nitric oxide synthase

ERK: Extracellular signal-regulated kinases

FAK: Focal adhesion kinase

GFP: Green fluorescent protein

GIRK: G-protein inwardly rectifying potassium channel

GTP: Guanosine triphosphate

HEK293: Human embryonic kidney cells

IFU: Infectious unit

IL: Interleukin

ILK: Integrin linked kinase

INF-γ: Interferon γ

iNOS: Inducible nitric oxide synthase

IRAS: Imidazoline receptor antisera selected

IRS: Insulin receptor substrate

JNK: c-Jun N-terminal kinase

LKB1: Liver kinase B1

LVH: Left ventricular hypertrophy

MAPK: Mitogen activated protein kinases

miRNA: Microribonucleic acid

mTOR: Mammalian target of rapamycin

NADPH: Nicotinamide adenine dinucleotide phosphate

NE: Norepinephrine

NF-κB: Nuclear factor κB

NO: Nitric oxide

NOS: Nitric oxide synthase

NPR: Natriuretic peptide receptor

PAK: p21-activated kinase

PC12: Rat pheochromocytoma cell line

PC-PLC: Phosphatidylcholine-selective phospholipase C

PDGF: Platelet derived growth factor

PGE2: Prostaglandin E2

PI3-K: Phosphatidylinositol-3 kinase

PKA: Protein kinase A

PKB: Protein kinase B

PKC: Protein kinase C

RAAS: Renin-angiotensin-aldosterone system

ROS: Reactive oxygen species

RVLM: Rostro-ventro lateral medulla

S1P: Sphingosine 1 phosphate

SERCA: Sarco(endo)plasmic Ca2+-ATPase

SHR: Spontaneously hypertensive rat

SNS: Sympathetic nervous system

TGF-β: Transforming growth factor-β

TNF-α: Tumor necrosis factor α

VEGF: Vascular endothelial growth factor

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# Chapter 1. Introduction

Cardiovascular pathologies, including hypertension, coronary artery disease, and heart failure, are the most common cause of death in the western world today (World Health Organization, 2014). According to the World Health Organization (WHO), more people die of cardiovascular diseases (CVD) yearly than any other cause or pathological condition, with approximately 17.3 million deaths due to CVDs in 2008, a number which is predicted to rise to 23.6 million by the year 2030 (Mathers & Loncar, 2006). In Canada, over 28.6% of total deaths per year are due to major cardiovascular diseases (Statistics Canada, 2012); in fact at every 7 minutes someone is suffering from acute myocardial infarct in Canada. The cost of cardiovascular diseases in Canada is of around $20.9 billion per year (Heart and Stroke Fundation of Canada, 2013), and this burden and cost will most likely increase with the aging of the population.

## 1.1 The burden of cardiovascular disease. Focus on hypertension and heart failure

High blood pressure is the leading risk factor for cardiovascular disease mortality, causing more than 7 million deaths every year worldwide (World Health Organization, 2014). Hypertension is also a growing problem. In Canada alone, the prevalence of hypertension has grown from 12.5% in 1988/1989 to 19.6% for the year 2007/2008; in the same year, in the age group of 75 to 79 years, 69.5% had hypertension (Robitaille et al., 2012). This increasing trend most likely will accelerate in time, due to the ageing of the population (Employment and Social Development Canada, 2014). In spite of all the efficacious antihypertensive treatments, one third of Canadian adults with hypertension have uncontrolled blood pressure (Campbell, McAlister, & Quan, 2013; Danaei et al., 2011).

Uncontrolled hypertensive patients develop left ventricular hypertrophy (LVH) (Ferrara, Vaccaro, Cardoni, Mancini, & Zanchetti, 2004). Using echocardiography, a sensitive method for LVH detection, a survey in Italy revealed that around 30 to 50% of hypertensive patients had LVH (Cuspidi et al., 2011). In hypertension, the degree of hypertrophy is directly

correlated to the severity of blood pressure elevation, being present in all patients with severe hypertension (Belenkov, Vikhert, Belichenko, & Arabidze, 1992). LVH is an independent cardiovascular risk factor (Chobanian et al., 2003) and lowering it using antihypertensive treatments lowers both cardiovascular and total mortality (Devereux et al., 2004).

A second major complication of hypertension is heart failure (Chobanian et al., 2003). The Framingham study showed that 75% of heart failure patients detected in the cohort had hypertension (defined as a blood pressure above 160 mmHg systolic or 95 mmHg diastolic). Additionally, heart failure risk is 10 times higher in hypertensive patients with LVH, linking these two complications (Kannel, Castelli, McNamara, McKee, & Feinleib, 1972). Heart failure has a very high mortality rate, with roughly 50% of patients dying during the first 5 years of diagnosis, even with modern treatments (Levy et al., 2002; Roger et al., 2004). Noteworthy, effective treatments of hypertension, which reduce LVH, also reduce the incidence of heart failure (Larstorp et al., 2012), pointing towards the importance of treatment of LVH as a preventive measure for heart failure.

Hypertension treatment usually involves the use of 2 or more medications from different pharmacological groups. Antihypertensive medications include thiazide diuretics, calcium channel antagonists, blockers of the renin-angiotensin-aldosterone system and peripheral or centrally acting antagonists of the sympathetic nervous system (Chobanian et al., 2003; Hackam et al., 2013) (see section 1.5 Current pharmacological treatment options for hypertension and heart failure for details). Despite this plethora of options, around 15% of treated patients will not have blood pressure within normal limits following the use of 3 or more different medication classes, thus being classified as having resistant hypertension (Daugherty et al., 2012; Khan et al., 2014).

Better understanding of possible interactions of systems that control blood pressure and/or cardiac function may lead to innovative ways of treatment to prevent cardiac pathologies progression, avoiding the pathophysiological consequences of heart failure.

A common feature in cardiovascular diseases, including hypertension and heart failure, is over-activity of the sympathetic nervous system (SNS) (Meredith et al., 1993; Schlaich et al., 2003). The SNS is implicated in the regulation of blood pressure and cardiac function.

However SNS over-activity contributes to the pathogenesis and consequences of cardiovascular diseases, by activating signaling events intimately linked to vascular and cardiac cell growth and death (Dorn, 2002; Osadchii, 2007). Imidazoline $I_1$-receptors in the brainstem are involved in the control of SNS activity, and consequently its deleterious effects on the heart, through mechanisms that inhibit the release of the neurotransmitter, norepinephrine (Bousquet, Feldman, & Schwartz, 1984; Raasch, Jungbluth, Schafer, Hauser, & Dominiak, 2003). Imidazoline $I_1$-receptors are also found in the heart (El-Ayoubi, Gutkowska, Regunathan, & Mukaddam-Daher, 2002; El-Ayoubi, Menaouar, Gutkowska, & Mukaddam-Daher, 2003, 2004) and are involved in atrial natriuretic peptide (ANP) release (Mukaddam-Daher, Lambert, & Gutkowska, 1997; Mukaddam-Daher, Menaouar, & Gutkowska, 2006), yet their cellular localization and function are not fully elucidated. Investigation of heart imidazoline $I_1$-receptor interaction with hypertension- and heart failure-associated control mechanisms may lead to new treatments of cardiovascular disease. We have enough evidence to propose that cardiac imidazoline $I_1$-receptors are involved in cardiac cell growth and death, thus may be a new therapeutic target.

## 1.2 The heart

For the ancient Egyptians, the heart was one of the main organs of the body, being the center of emotions and intelligence as well as responsible for transport of blood (Ziskind & Halioua, 2004). This spiritual point of view has evolved towards the present view of the heart as a structure with multiple and tightly controlled functions on the cardiovascular system. The heart is involved in the maintenance of blood pressure and blood flow towards the different organs (Silverthorn, 2013; West, 1990). It acts as a pump that propels blood towards the vasculature, and as an endocrine organ that produces natriuretic hormones involved in blood pressure and volume control (de Bold, Borenstein, Veress, & Sonnenberg, 1981). Furthermore, both pump and endocrine activities are under homeostatic control of the sympathetic nervous system, as well as circulating and locally produced neurohormones and cytokines (Silverthorn, 2013; West, 1990). Abnormalities in the control mechanisms lead to

altered heart structure and function and consequently impair cardiac function (Cacciapuoti, 2011; Chaggar, Malkin, Shaw, Williams, & Channer, 2009).

### 1.2.1 The heart as a pump

The heart contracts and relaxes in a cyclical way (Iaizzo, 2009; West, 1990). During contraction, the phase known as systole, the heart contracts, pumping into the aorta a quantity of blood. This pumping function works against a resistance, generating pressure, an effect that maintains blood flow. Blood pressure is the product of flow (or cardiac output) and the resistance to flow, also known as arterial resistance (Fukuta & Little, 2008; Iaizzo, 2009).

Cardiac output is an indicator of total blood flow, because all the blood that leaves the heart flows through the tissues. Since the needs of the body are variable, blood flow (cardiac output) adapts to accommodate different situations. Cardiac output, a common measure of cardiac function, directly varies with the ejected volume per contraction (stroke volume, SV) and by the number of cardiac cycles (or beats per minute, heart rate, HR) in a relationship shown in the formula: CO = SV x HR (reviewed by West, 1990) (West, 1990). This relationship that appears simple is extremely complex, as many factors are involved in the regulation of each parameter.

Heart rate is under the control of the autonomic nervous system, decreasing by active parasympathetic nervous system and increasing by active SNS and epinephrine (Bonow, Mann, Zipes, Libby, & Branwald, 2012). Increased heart rate may be maladaptive under pathological circumstances (Bonow et al., 2012; Brunton, Chabner, & Knollmann, 2011), and thus could be a target for treatment with sympatholytics.

On the other hand, stroke volume varies with the amount of blood that returns from the tissues to the heart. This amount of blood (venous return) is controlled by muscle pump, respiratory pump as well as by sympathetic innervation of veins, which constricts the veins, squeezing more blood out of them and into the heart (Levy, Koeppen, & Stanton, 2006; Silverthorn, 2013). With a larger ventricular volume at the beginning of the next contraction (end diastolic volume, EDV), the muscle fibers stretch, and then contract more forcefully, ejecting more

blood. The degree of myocardial stretch before contraction begins is called the preload on the heart, because this stretch represents the load placed on cardiac muscles before they contract. Changes in preload change contractility in the same sense, so, an increase of the preload will increase contractility and stroke volume, a phenomenon known as Frank-Starling mechanism (Bombardini, 2005; Shiels & White, 2008). The Frank-Starling mechanism fails in heart failure, when the heart is unable to respond to preload changes, thus elevating the pressure in the venous system, and increasing the symptoms of congestion (Kemp & Conte, 2012). This is the rationale for the use of medications that reduce preload, like diuretics and some venous dilators, in the current in treatment of heart failure (Hunt et al., 2009).

Stroke volume is also influenced by afterload, which is the combined load of EDV and arterial resistance created by blood filling the arterial system during ventricular contraction. For all practical purposes, unless there is an obstruction to blood flow, afterload is directly related to the systolic pressure (Fukuta & Little, 2008; Silverthorn, 2013) and clinically, arterial blood pressure is often used as an indirect indicator of afterload. As blood pressure is inversely related to the vessel radius, a small decrease in vessel radius will induce marked increase in blood pressure that increases afterload and might affect cardiac output (Levy et al., 2006; West, 1990).

Vessel radius (and thus afterload) is mainly controlled by the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS). Through corresponding release of neurohormones and hormones, these vasoconstrictive, anti-natriuretic, anti-diuretic and growth promoting systems influence vessel radius and tone and consequently cardiac function (Kemp & Conte, 2012; Silverthorn, 2013; West, 1990). In cardiovascular pathologies, such as hypertension or heart failure, afterload is usually elevated, increasing cardiac wall stress and work (Kemp & Conte, 2012; Lam et al., 2013). This is the rationale for treatment with medications that act through blocking the activities of the RAAS and the SNS (Hackam et al., 2013; Hunt et al., 2009).

Ventricular contraction occurs due to the entry from the extracellular space of small amounts of calcium, which in turn induce the release of large amounts of calcium from the endoplasmic reticulum of cardiomyocytes, permitting the cross-link between actin and myosin (Bombardini, 2005). Calcium concentration and myofilament sensitivity to calcium can be

modified by mechanical (preload) or endocrine/paracrine factors, including catecholamines, which increase the available calcium, thus increase contractility. Medications that modify sympathetic tone also modify contractility (Bombardini, 2005; Brunton et al., 2011; Shiels & White, 2008; Silverthorn, 2013).

The second component of cardiac function, relaxation (called lusitropism), depends on the active extrusion of calcium (including its rate). Once the cytoplasmic calcium concentration decreases, the cross-link between actin and myosin disappears and the tension decreases, starting the diastole (Bombardini, 2005; Levy et al., 2006; West, 1990). Relaxation also depends on the static properties of the myocardium, specifically the amount of collagen in the tissue. In pathological conditions, the lack of relaxation can disturb ventricular function by impeding the filling of the heart (Bonow et al., 2012; Diez et al., 2002).

From the above description, it is clear that the *in vivo* determinants of cardiac function act in concert to maintain cardiac output following a wide range of changes, and that the SNS plays an important role in the neurohormonal control of each of these determinants (Figure 1).

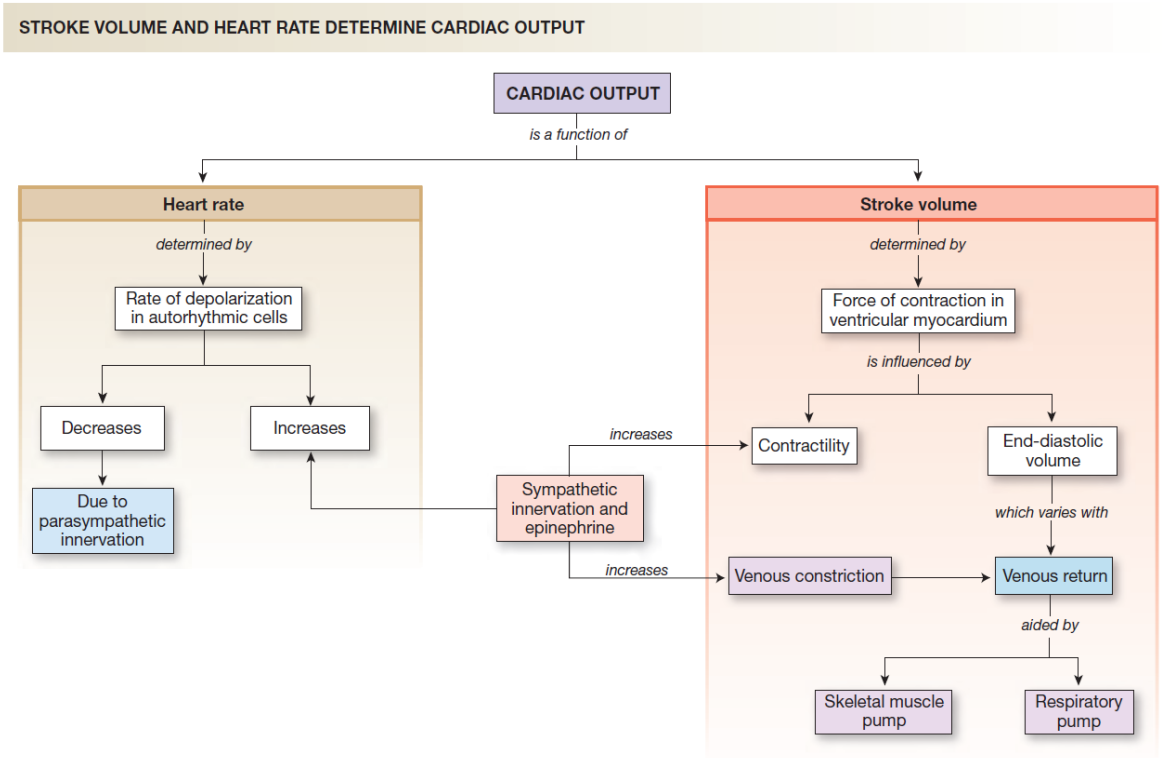


Figure 1: Schema showing the central role of the sympathetic nervous system in the control of cardiac output (Silverthorn, 2013).

## 1.2.2 The endocrine heart

The heart itself is in a privileged position to sense the hemodynamic conditions present in the circulatory system. This sensory system responds to volume changes by secreting diverse substances, including cardiac natriuretic hormones and other paracrine/autocrine factors like adrenomedullin and endothelin-1 (Ogawa & de Bold, 2014).

Adrenomedullin is mostly produced in the adrenal medulla, but it's also detectable in the heart, both in atria and ventricles (Ichiki et al., 1994). Adrenomedullin can act locally to decrease angiotensin II-induced cardiomyocyte protein synthesis, a marker of hypertrophy, thus cardiac production of adrenomedullin may counter-regulate cardiac hypertrophy (Ogawa & de Bold, 2014; Tsuruda et al., 1998).

Endothelin-1, the most potent vasoconstrictor known, is mostly produced in vascular endothelial cells, but also detected in other tissues, including the heart, in cardiomyocytes,

fibroblasts and endothelial cells (Sakurai et al., 1991; van Wamel, Ruwhof, van der Valk-Kokshoom, Schrier, & van der Laarse, 2001; Yanagisawa et al., 1988). Endothelin-1 induces cardiomyocyte hypertrophy and fibrosis (Drawnel, Archer, & Roderick, 2013), thus it is involved in the development of LVH. On the other hand, endothelin-1 induces cardiac secretion of natriuretic peptides (Horio, Kohno, & Takeda, 1993), a counter-regulatory protective effect.

The natriuretic peptide family, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), are structurally and functionally related peptide hormones that are involved in blood pressure regulation and body fluid homeostasis under normal and pathological conditions, such as heart failure and hypertension (Ogawa & de Bold, 2014). They are primarily of cardiac origin, but also produced by several extracardiac tissues, including the gastrointestinal tract and the brain (Gower et al., 1994; Teran, Rodriguez-Iturbe, Parra, & Gutkowska, 1991). In the heart, ANP is synthesized and stored in granules in cardiac atrial cardiomyocytes (de Bold et al., 1981). ANP release into the circulation is continuous, increasing in response to stimuli that result in atrial distension (Dietz, 2005; McGrath & de Bold, 2005; Thibault, Amiri, & Garcia, 1999). ANP secretion is stimulated by increasing venous return or increasing aortic pressure, as well as by vasoactive factors, neurotransmitters, and pro-inflammatory cytokines (Interleukin (IL)-1β, IL-6, tumor necrosis factor-α) (Ambler & Leite, 1994; Dietz, 2005; Mukaddam-Daher, 2006; Schiebinger & Greening, 1992). Nitric oxide, calcitonin gene-related peptide, and histamine inhibit ANP release (Dietz, 2005; Li et al., 2003; Piao, Cao, Han, Kim, & Kim, 2004).

BNP is co-stored with ANP in some atrial and ventricular granules (Nakamura et al., 1991); it is believed that BNP is secreted predominantly from cardiac ventricles (myocytes and fibroblasts), yet most of regulated BNP secretion, in response to increased ventricular stretch and endothelin, happens in the atria (Ogawa & de Bold, 2014; Ogawa, Vatta, Bruneau, & de Bold, 1999). BNP levels are increased in cardiovascular diseases, including ischemia, arrhythmias, fibrosis, cardiac hypertrophy, coronary endothelial dysfunction and heart failure (Mukaddam-Daher, 2006; Ogawa & de Bold, 2014). CNP is synthesized and secreted by the coronary endothelium and by cardiac myocytes and fibroblasts (Del Ry et al., 2011). Endothelial production of CNP is enhanced in response to shear stress, pro-inflammatory

cytokines and lipopolysaccharide, and inhibited by insulin and VEGF. Myocardial production of CNP is increased in chronic heart failure. Plasma CNP, which is normally very low or undetectable, is significantly increased in patients with chronic heart failure, cirrhosis, and sepsis (reviewed by Mukaddam-Daher, 2006)(Mukaddam-Daher, 2006).

The natriuretic peptides elicit a number of vascular, renal and endocrine effects that help maintain blood pressure and extracellular fluid volume, and are involved in neuronal cardiac regulation, influencing contractility and beating rate (Antunes-Rodrigues, de Castro, Elias, Valenca, & McCann, 2004; Mukaddam-Daher, 2006). Circulating ANP and BNP can produce hypotensive effects, particularly in elderly patients (Hausdorff, Clark, Shannon, Elahi, & Wei, 1995), due to their diuretic, natriuretic, and vasodilatory properties (Koda, Sakamoto, & Ogawa, 2005; Melo, Steinhelper, Pang, Tse, & Ackermann, 2000). These effects are attained by direct inhibition of renal tubular sodium reabsorption, particularly in the distal tubule (Zhao, Pandey, & Navar, 2010), by inhibition and counteracting the effects of the sympathetic nervous system and the renin-angiotensin-aldosterone system, as shown in KO mouse models and in patients (Kasama et al., 2007; Melo et al., 2000), and by action on brain regulatory sites to suppress thirst, and inhibit vasopressin and ACTH release and sympathetic outflow (Antunes-Rodrigues et al., 2004). Natriuretic peptides inhibit the production of endothelin and secretion of renin and aldosterone. CNP is considered as a neuropeptide and an endothelium-derived autocrine/paracrine regulator exerting a hypotensive effect, positive inotropic action, but no significant diuretic or natriuretic actions; however, it inhibits aldosterone synthesis in a similar manner to ANP & BNP (reviewed by Mukaddam-Daher, 2006)(Mukaddam-Daher, 2006).

The important role of ANP in chronic blood pressure regulation has been shown in transgenic mouse model overexpressing ANP, where elevated plasma ANP was associated with a 25-30 mmHg blood pressure reduction, an opposite effect is observed in ANP KO, where blood pressure is around 30 mmHg higher when compared to wild type animals (Melo et al., 2000). Knockout mice with a homozygous disruption of the pro-ANP gene (-/-) are incapable of producing ANP and are hypertensive relative to their wild-type siblings, developing salt-sensitive hypertension after prolonged feeding of a high salt diet, in part due to a synergistic interaction between angiotensin II and sympathetic nerve activity, and their inability to reduce

plasma renin activity (Melo et al., 1999). The mechanism mediating the chronic relaxant effect of ANP is indirect, since the resistance vasculature is relatively insensitive to direct cGMP-mediated relaxation by ANP. Melo et al., have shown in ANP knockout mouse that the chronic hypotensive effect of ANP is mediated by attenuation of tonic cardiovascular sympathetic tone (Melo et al., 1999).

The actions of natriuretic peptides ANP and BNP are mediated by a common transmembrane cell surface guanylyl cyclase receptor (NPR-A), and CNP through NPR-B, which act through generation of cGMP and subsequent activation of cGMP-dependent protein kinase G (PKG) (Ogawa & de Bold, 2014). All 3 peptides bind to a more abundantly expressed receptor (NPR-C), which lacks the cytoplasmic guanylyl cyclase domain. NPR-C promotes the peptide clearance from the circulation and mediates inhibition of endothelin release and antagonism of the renin-angiotensin-aldosterone via Giα and inhibition of adenylyl cyclase/cyclic adenosine monophosphate (cAMP) (El Andalousi, Li, & Anand-Srivastava, 2013; Li, Sarkar, Brochu, & Anand-Srivastava, 2014). Natriuretic peptide receptors are ubiquitous, present in kidneys, vasculature, lungs, brain, thymus, macrophages etc., and recently demonstrated in the heart, where local actions of natriuretic peptides may occur (Lin, Hanze, Heese, Sodmann, & Lang, 1995). In the heart, the mRNAs for all three NPRs are found in fibroblasts with 80% of the NPR-C subtype. NPR-A is abundant in ventricular myocytes, whereas NPR-B is abundant in fibroblasts and endothelial cells (Lin et al., 1995).

Based on the previously described findings, it is clear that the heart protects itself through its endocrine functions, both systemically, by regulating blood pressure, and locally, by counter-regulating hypertrophic and fibrotic stimuli.

## 1.3 The continuum hypertension-LVH-heart failure

During exercise, the heart is required to pump facing increases in preload (for example in swimming), afterload (weightlifting), or both (rowing) (Ekblom & Hermansen, 1968; Pelliccia, Maron, Spataro, Proschan, & Spirito, 1991; Weeks & McMullen, 2011). The ventricle increases its force of contraction to maintain adequate stroke volume, increasing the muscle's need for oxygen and ATP production. Also, neurohormonal compensatory

mechanism are activated, causing cardiovascular stress (reviewed by Weeks and McMullen, 2011) (Weeks & McMullen, 2011). However, normal, short term cardiovascular stresses, such as bursts of exercise, have beneficial effects on the cardiovascular system and are an integral part of preventive and treatment strategies in hypertension and heart failure (Chobanian et al., 2003; Hackam et al., 2013; Hunt et al., 2009). In contrast, chronic cardiovascular stress (such as chronically increased afterload as occurs in hypertension) cause myocardial cells to hypertrophy, resulting in increased thickness of the ventricular wall, leading to maladaptive changes such LVH (Weeks & McMullen, 2011). In hypertension, the degree of hypertrophy is directly correlated to the severity of blood pressure elevation, being present in all patients with severe hypertension (Belenkov et al., 1992).

The hypertrophic response of the heart is a dynamic process that involves progressive changes in gene expression that creates structural, hemodynamic, and cellular alterations (Carreno, Apablaza, Ocaranza, & Jalil, 2006; Weeks & McMullen, 2011). LVH involves cardiomyocyte hypertrophy, cardiac fibroblast proliferation, increased synthesis and deposition of collagen, and progression of interstitial and perivascular fibrosis (Cacciapuoti, 2011). Initially, these alterations contribute to lowering of the ventricular wall stress and preservation of contractile function; a stage known as adaptive or compensatory. Eventually, the remodeling process leads to ventricular stiffness, secondary to a decrease in the active extrusion of calcium after the contraction of the myocardium (i.e. reduced relaxation), and alterations of the static properties of the myocardium, secondary to the increased amount of collagen in the tissue. Cardiac stiffness can impede the filling of the heart, hence, results in reduced force of contraction (Frank-Starling mechanism) and subsequent reduction in ejection fraction, stroke volume, and cardiac output (Bonow et al., 2012; Kemp & Conte, 2012).

The kidneys respond to reduced cardiac output and subsequently reduced renal perfusion by altering their hemodynamic milieu through a number of physical and neurohumoral mechanisms, including activated renin-angiotensin-aldosterone system (RAAS) and vasopressin, as well as stimulated catecholamines and renal nerve activity. These vasoconstrictive and sodium and water retaining mechanisms lead to volume expansion and to further deterioration in cardiac performance (Bonow et al., 2012; Kemp & Conte, 2012). The result is a failing heart that cannot pump out sufficient blood to supply the needs of the body and progressive volume retention,

which results in systemic and pulmonary edema, as well as progressive apoptosis and fibrosis (Kemp & Conte, 2012; Ward, Crossman, & Cannell, 2011). Exaggerated apoptosis, a mode of cell death in which the cell participates in its own demise, may account for the loss of contractile cardiomyocytes in the hypertensive left ventricle, which may further progress towards heart failure (Diez, Fortuno, & Ravassa, 1998; Ikeda, Hamada, & Hiwada, 1999; Li, Bing, Long, Robinson, & Lakatta, 1997).

In fact, LVH is linked to unfavorable prognoses and various deleterious sequelae of cardiovascular diseases, with conditions, such as coronary heart disease, stroke, congestive heart failure and sudden death, being aggravated by LVH (Devereux et al., 2004; Larstorp et al., 2012). Importantly, LVH can often be reversible. It is not surprising, therefore, that numerous therapeutic approaches have been pursued with the aim of regressing/preventing LVH and, hence, reducing both cardiovascular and total mortality (Devereux et al., 2004).

## 1.4 Factors involved in the development of LVH and heart failure

Similar to all cells in the body, cardiomyocytes and fibroblasts sense and respond to their mechanical and neurohormonal environment (Bernardo, Weeks, Pretorius, & McMullen, 2010; Haggart, Ames, Lee, & Holmes, 2014). The mechanical environment includes mechanical stretch as well as the composition and stiffness of the extracellular matrix (ECM), a network of cellular and extracellular constituents that comprises the left ventricular myocardium (Jane-Lise, Corda, Chassagne, & Rappaport, 2000). *In vivo* cardiac remodelling, including cytoskeleton reorganization, results from a combination of the effects of mechanical and biochemical factors in close interaction with extracellular matrix (ECM) proteins, integrins, and proteins secreted locally, such as metalloproteinases (Bernardo et al., 2010).

### 1.4.1 Mechanical Factors

Pressure overload in hypertension or volume overload, for example, in mitral valve insufficiency, result in mechanical stretch, which influences many basic cellular processes such as growth, remodeling, apoptosis, and gene expression (Liu et al., 1992; Sadoshima,

Jahn, Takahashi, Kulik, & Izumo, 1992; Wilson, Mai, Sudhir, Weiss, & Ives, 1993), as well as differentiation (Reusch, Wagdy, Reusch, Wilson, & Ives, 1996), rearranged cytoskeleton or focal contacts (Carver, Nagpal, Nachtigal, Borg, & Terracio, 1991; Davies, Robotewskyj, & Griem, 1994; Girard & Nerem, 1995; Moore et al., 1994), or altered composition of the extracellular matrix (Jane-Lise et al., 2000).

Mechanical stretch induces protein synthesis and hypertrophy, both in isolated cardiomyocytes and in papillary muscle preparations (Haggart et al., 2014; Lammerding, Kamm, & Lee, 2004; Wang, Wu, Cheng, & Shyu, 2013), induces secretion or synthesis of bioactive molecules from cardiomyocytes (Shyu, 2009), and increases production of extracellular matrix proteins by fibroblasts, the principal cell type responsible for extracellular matrix synthesis during growth and pathophysiological conditions (Jugdutt & Amy, 1986; Kuwahara et al., 2003; Weber, Brilla, & Campbell, 1992).

Mechanical stretch is detected by cardiac cells through multiple mechanisms, including mechanosensitive ion channels, which are stretch activated channels that permit the handling of sodium and calcium (Bonow et al., 2012; Lammerding et al., 2004) as well as by cell surface adhesion receptors, termed integrins (Lammerding et al., 2004).

Integrins form the primary link between extracellular matrix ligands and cytoskeletal structures, which is important for maintaining the architecture of tissues (Hynes, 1992; Lammerding et al., 2004; Ruoslahti, 1991). In myocardial tissue, integrins play a role in transmitting and distributing the mechanical force generated by the contraction of each myocyte to the extracellular matrix, and preventing myocytes from overstretching by elevated tension (Borg, Johnson, & Lill, 1983; Winegrad & Robinson, 1978).

Integrins are a family of transmembrane glycoproteins that function as mechanotransducers, converting mechanical forces to biochemical signals. The integrin large extracellular domain binds directly to ECM proteins, like collagen or fibronectin, among others (Evans & Calderwood, 2007; Lammerding et al., 2004), playing a crucial role in ECM organization. The integrin cytoplasmic tail does not have an intrinsic enzymatic activity (Lammerding et al., 2004), but binds to cytoskeletal proteins, such as F-actin, through the interaction of integrin tails with talin (Ross, 2002; Ross et al., 2013), as well as to signalling proteins, including

integrin linked kinase (ILK), focal adhesion kinase (FAK), the small guanosine triphosphate (GTP) binding proteins Rho and Rac1, with downstream activation of mitogen activated protein kinases (MAPK), p21-activated kinase (PAK), nuclear factor-κB (NF-κB) and Akt/protein kinase B (PKB), pathways that have been linked to hypertrophy, proliferation, and apoptosis in different cell types, including cardiomyocytes and fibroblasts (Figure 2) (Aikawa et al., 2002; Bettink et al., 2010; Krishnamurthy, Subramanian, Singh, & Singh, 2007; Lammerding et al., 2004; Ross, 2002).

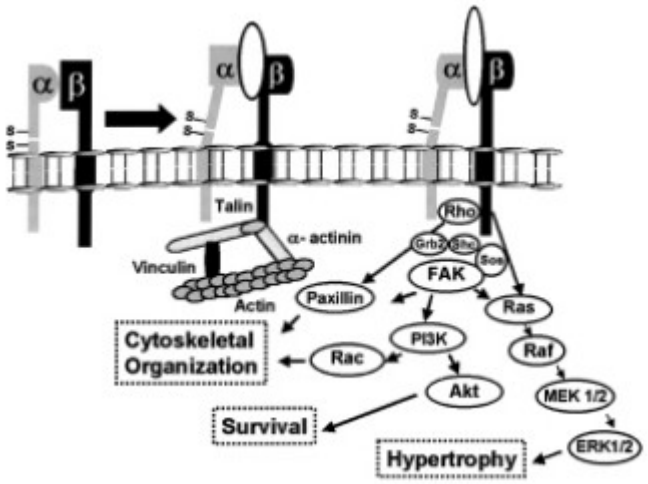


Figure 2: Mechanisms involved in integrin-mediated mechanotransduction in cardiomyocytes. Of note the link with cytoskeletal proteins and signalling pathways inducing hypertrophy. For details see text (Ross, 2002).

Rac1 is a small guanosine triphosphatase catalytic subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Hordijk, 2006). Rac1 activation increases intracellular reactive oxygen species (ROS), such as superoxide ($\cdot O_2-$) and $H_2O_2$, into which $\cdot O_2$ is rapidly converted. ROS are important mediators of cellular signal transduction cascades such as proliferation, migration, and apoptosis (Maack et al., 2003). Excess ROS production damages DNA, protein and lipids, causing cardiac cell death. Activation of Rac1 and its effector protein NADPH oxidase can also regulate proliferative signals through mechanisms that include NF-κB stimulation (Hordijk, 2006), mediated by increasing intracellular ROS or through phosphatidylinositol-3 kinase (PI3-K)/Akt. The Rac1–ROS signaling pathway is a crucial mediator of cardiovascular disease (Hordijk, 2006). Inhibition of Rac1 by statins

decreases NADPH oxidase-related production of ROS in cardiomyocytes and reduces cardiac hypertrophy (Maack et al., 2003), demonstrating the importance of Rac1 activity in cardiac pathologies. The direct involvement of Rac1 in cardiovascular disorders has been confirmed by both cardiac-specific transgenic and knockout mice. Mice overexpressing the constitutively active form of Rac1 clearly evidence a dramatic cardiomyopathy phenotype (Sussman et al., 2000). In contrast, in cardiac-specific Rac1 knockout mice, infusion of angiotensin II increases systolic blood pressure but does not induce cardiac hypertrophy in association with the inhibition of NADPH oxidase-dependent generation of $O_2^-$ and NF-κB transcriptional activity (Satoh et al., 2006).

Mammalian integrins comprise 18α and 8β subunits (Ross & Borg, 2001). Cardiac myocytes predominantly express β1 integrin, the main integrin subtype that mediates adhesion to the extracellular matrix (Ross & Borg, 2001). β1 integrin heterodimers significantly contribute to cardiomyocyte adhesion to the ECM, and inhibition of β1 integrin-mediated adhesion leads to ventricular dilatation of the normal heart, and plays a significant role in the progression of adverse myocardial remodeling that leads to heart failure (Stewart, Gardner, Brower, & Janicki, 2014). In addition, β1 integrin signaling protects cardiomyocytes against β-adrenergic receptor-stimulated apoptosis *in vitro* and left ventricular remodeling and myocyte apoptosis *in vivo* (Krishnamurthy et al., 2007). *In vivo* infusion with β-adrenergic receptor agonist isoproterenol increases cardiomyocyte apoptosis in mice with partial knock-out of β1 integrin subunit, in association with reduced ventricular function and overactivation of c-Jun N-terminal kinase (JNK), when compared to wild type animals subjected to the same treatment (Krishnamurthy et al., 2007). Over-expression of β1 integrins, in neonatal rat ventricular myocytes, enhances the hypertrophic effects of α1-adrenergic stimulation, and inhibition of β1 integrin function and signaling reduces the hypertrophic response (Ross et al., 1998).

The α5 integrins, on the other hand, modulate cardiac neural crest proliferation and survival, and are required for cardiac morphogenesis and, in particular, for the formation of the cardiac outflow tract (Mittal, Pulina, Hou, & Astrof, 2010, 2013). The expression of α1, α3 and α5 integrins is low in normal cardiomyocytes, but is upregulated by myocardial infarction in rats (Nawata et al., 1999). Integrin α5 subunit is also over-expressed in LVH (Ross, 2002). The expression of integrin α5 subunit is regulated by cardiovascular disease-activated growth

factors and cytokines (Nawata et al., 1999). Platelet derived growth factor (PDGF) upregulates the expression of the α5 integrin subunit in cardiomyocytes. Transforming growth factor-β (TGF-β) and inflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) or interferon γ (IFN-γ) enhance the expression of both α1 and α5 integrin subunits (Gailit, Xu, Bueller, & Clark, 1996; Nawata et al., 1999). In fibroblasts, the expression of α5 integrin is relatively higher in cells obtained from exercised rat hearts and lower in those from hypertensive hearts when compared to control, and these cells migrate on fibronectin at higher and lower rates respectively, demonstrating a direct correlation between the expression of α5 integrin and fibroblast migration (Burgess, Terracio, Hirozane, & Borg, 2002).

The β1 integrin forms dimers with multiple α subunits. Each specific dimer serves as a receptor for a specific extracellular matrix protein. For example α1β1 and α2β1 integrin complexes can bind to collagen and laminin, α4β1 and α5β1 to fibronectin, while α3β1 binds collagen, laminin, and fibronectin (Ross & Borg, 2001).

The α5β1 integrin (fibronectin receptor) is present in cardiomyocytes, but predominantly in fibroblasts (Clark et al., 2005). It supports migration in several cell types (Alahari, Lee, & Juliano, 2000) and its mutation or knock-out results in reduction or abolition of migration (Huttenlocher, Sandborg, & Horwitz, 1995). Increased expression of α1β1 and α5β1 heterodimers are found in association with increased adhesion during the compensatory hypertrophy secondary to renovascular hypertension (Terracio et al., 1991).

Over-expression of α5β1 integrin protects cell lines against apoptotic stimuli, in part by modulating the expression of the anti-apoptotic protein Bcl2, by activating the PI3-K/Akt pathway (Figure 3) (Lee & Ruoslahti, 2005; Lee & Juliano, 2000).

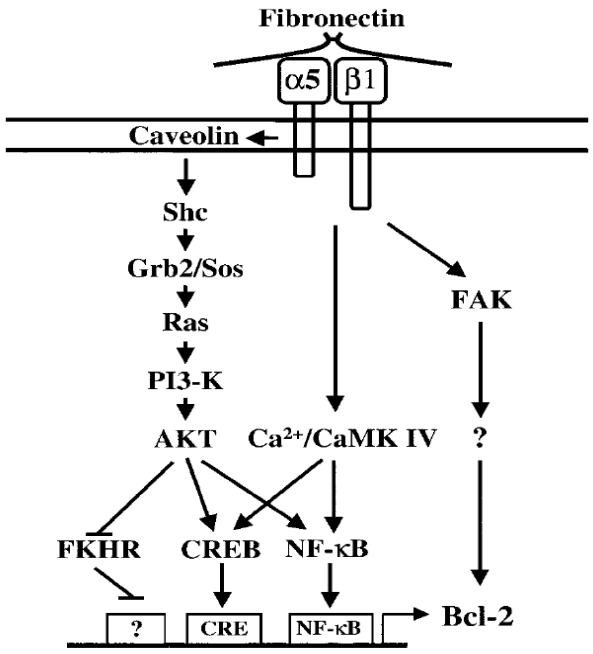


Figure 3: Signalling pathways stimulated by α5β1 integrin leading to an increase in Bcl-2 expression. Grb2: Growth factor receptor-bound protein 2; Ras: Rat sarcoma protein; PI3-K: Phosphatidylinositol-3 kinase; CREB: cAMP response element-binding protein; FKHR: Forkhead in rhabdomyosarcoma protein; FAK: Focal adhesion kinase; CaMK IV: Calcium calmoduline dependant kinase IV (Lee & Ruoslahti, 2005).

Furthermore, modulation of the α5β1 receptor ligand, fibronectin, is implicated in cardiac remodeling. Loss of fibronectin leads to further deterioration of cardiac function in a mouse model of myocardial infarction, due to impaired wound healing and reduced progenitor cell recruitment (Konstandin, Toko, et al., 2013). In contrast, genetic conditional ablation of the fibronectin gene in adult mice blunts cardiomyocyte hypertrophy upon pressure overload induced by transverse aortic constriction, and delays development of heart failure and improves survival (Konstandin, Volkers, et al., 2013). Noteworthy, induction of fibronectin expression upon myocardial infarction is very quick, while in the pressure overload model, fibronectin continuously increases over time at a lower level of expression (Konstandin, Toko, et al., 2013; Konstandin, Volkers, et al., 2013). Accordingly, it appears that the duration and intensity might impact the ultimate outcome of fibronectin expression.

Taken together, these studies show that mechanical overload leads to hypertrophic remodeling, mediated by mechanotransducing integrins, including α5β1 fibronectin receptor

and activation of Rac1 and subsequent ROS production. Of interest to our studies, imidazoline $I_1$-receptor/nischarin associates with the α5 subunit of the α5β1 integrin and reduces cell proliferation and migration through inhibition of Rac1 (Alahari et al., 2000). The association with integrins puts imidazoline $I_1$-receptors/nischarin at the heart of ECM control and cardiac remodelling, a function that may go beyond its established central and peripheral sympatholytic effect.

## 1.4.2 Biochemical Factors

The hypertensive heart undergoes significant structural changes that occur in response to biochemical stress, induced by activated sympathetic nervous system (SNS), the renin-angiotensin-aldosterone system (RAAS), growth factors, and pro-inflammatory cytokines. In addition to their hemodynamic activity, these factors initiate and maintain left ventricular hypertrophy through a complex array of signaling events, with cross-talk and positive and/or negative interaction, and contribute to regression to a fetal program of gene expression (reviewed by Cacciapuoti, 2001; Sugden and Clerk, 1998)(Cacciapuoti, 2011; Sugden & Clerk, 1998). On the other hand, the heart produces natriuretic peptides (ANP and BNP), vasodilatory, diuretic and natriuretic hormones, and more importantly, with anti-hypertrophic, anti-proliferative, anti-inflammatory, and sympatholytic actions (reviewed by Ogawa and de Bold, 2014) (Ogawa & de Bold, 2014).

### 1.4.2.1 The sympathetic nervous system

The sympathetic nervous system (SNS) plays an important role in the regulation of blood pressure and cardiac function (Grassi, Seravalle, & Quarti-Trevano, 2010). It is involved in minute-to-minute control of blood pressure because it responds to pressure alterations within seconds (Silverthorn, 2013; West, 1990). SNS also influences long term pressure control through activation of the renin-angiotensin-aldosterone system (RAAS). Renal nerves stimulate renin release and positively enhance angiotensin II, which directly stimulates muscle sympathetic nerve activity and facilitates adrenergic sympathetic transmission (Brunton et al., 2011; Silverthorn, 2013).

Sympathetic nervous activity is regulated in centers in the brainstem and transmitted to organs and blood vessels that are innervated by sympathetic nerve endings (Brunton et al., 2011). Overactivation of the sympathetic outflow to the heart, kidneys and skeletal muscle vasculature is commonly present in young patients with essential hypertension, where it leads to the development of cardiac risk factors, such as development of left ventricular hypertrophy, predisposing to ventricular arrhythmias, increasing insulin resistance and accelerating atherogenesis (Grassi et al., 2010). The level of sympathetic drive to the heart is a major determinant of prognosis in patients with hypertension and heart failure, and a decrease in heart rate and nerve activity by treatments that directly block or oppose the sympathetic nervous system has been shown to be beneficial for long-term prognosis in these patients (Frohlich, Gonzalez, & Diez, 2011).

The effects of the SNS are mediated by the neurotransmitter norepinephrine, a catecholamine synthesized in the neurons from tyrosine. The rate-limiting step for this synthesis is in the transformation of tyrosine to L-dopa, by the enzyme tyrosine hydroxylase (Figure 4) (Becker, 2012; Brunton et al., 2011). Norepinephrine is then stored at nerve endings until released by exocytosis upon stimulation. At the adrenal medulla, which is considered as a sympathetic ganglion, norepinephrine undergoes methylation producing epinephrine, which has similar actions to norepinephrine, but binds with similar affinity to all adrenergic receptor subtypes, while norepinephrine binds with higher affinity to β1- than β2- adrenergic receptors (Brunton et al., 2011).

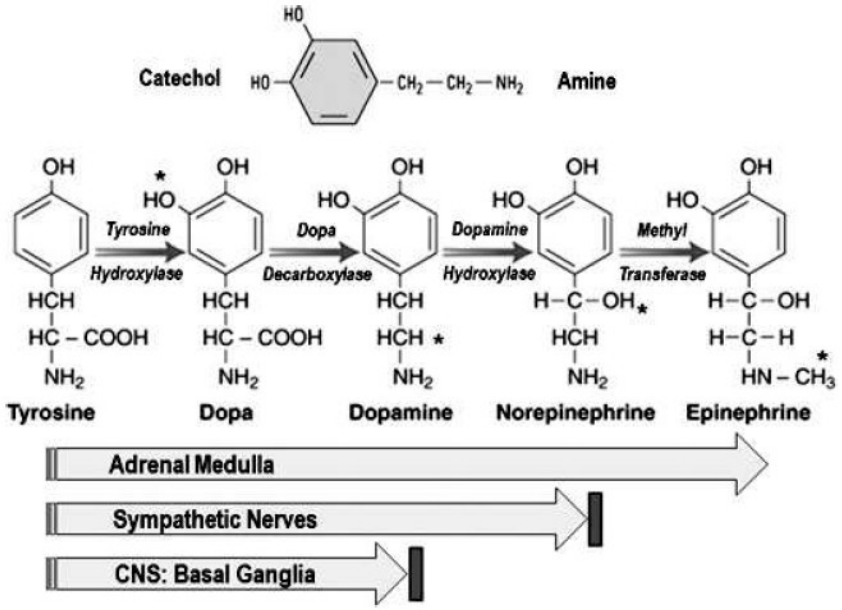


Figure 4: Steps involved in catecholamine synthesis. Of note, different catecholamines are produced in different sites depending on the local availability of enzymes (Becker, 2012).

Norepinephrine signals via its interaction with α- and β-adrenergic receptors, a family of G protein-coupled receptors (Becker, 2012; Brunton et al., 2011). Norepinephrine controls its own release by acting on α2-adrenergic receptors present on the pre-synaptic membrane, inhibiting further release (Brunton et al., 2011; Kalant, Grant, & Mitchell, 2007). Norepinephrine release is also inhibited by activation of imidazoline $I_1$-receptors, non-adrenergic receptors expressed in brainstem medulla and pre-synaptic membranes (Chan, Burke, Zhu, Piletz, & Head, 2005; Molderings & Gothert, 1998). The effects of norepinephrine are terminated through two mechanisms: reuptake by the norepinephrine transporter, and diffusion followed by degradation by extracellular enzymes (Brunton et al., 2011; Kalant et al., 2007).

The released norepinephrine causes vasoconstriction through binding to vascular α1-adrenergic receptors (Hosoda et al., 2005; Zacharia, Hillier, & MacDonald, 2004). In the kidney, stimulated SNS activity/norepinephrine constricts afferent arterioles and reduces glomerular filtration rate by decreasing blood flow into glomerular capillaries (DiBona, 2002).

On the heart, norepinephrine acutely increases heart rate and contractility by activating β1-adrenergic receptors, the main adrenergic receptor subtype (80%) present in the heart, specifically in cardiomyocytes (Brunton et al., 2011; Singh, Xiao, Remondino, Sawyer, & Colucci, 2001). Short term (10 minutes) activation of the β1-adrenergic receptor increases cardiomyocyte contractility by inducing an increase in calcium transients, which are dependent on the activation of protein kinase A (PKA) (Wang et al., 2004). Longer exposure (24 h) to β1-adrenergic stimulation of isolated adult rat cardiomyocytes switches the increase in contractility and calcium transients from PKA dependency towards dependency on the activity of the calcium/calmodulin-dependent protein kinase II (CaMKII), an enzyme that has also been linked to adrenergic-induced apoptosis (Wang et al., 2004; Zhu et al., 2003).

In heart failure, the expression of β1-adrenergic receptor in cardiomyocytes is down-regulated and uncoupled, leading to a decrease in contractility (Bristow et al., 1986; Lamba & Abraham, 2000; Osadchii, 2007; Parati & Esler, 2012). The down regulation of β1-adrenergic receptors gives importance to the activity of β2- and α1-adrenergic receptors (Lamba & Abraham, 2000; Osadchii, 2007). During β1-adrenergic receptor down regulation, α1-adrenergic receptors sustain adrenergic function (Lamba & Abraham, 2000).

*In vivo* and *in vitro* studies demonstrate that prolonged exposure to norepinephrine causes ventricular hypertrophy, with cardiomyocyte hypertrophy and death, fibroblast proliferation, and an increase in collagen accumulation (Bhambi & Eghbali, 1991; Lamba & Abraham, 2000; Singh et al., 2001), increasing the risk of cardiac failure. In genetically engineered mice, deletion of α2A- and α2C-adrenergic receptors leads to cardiac hypertrophy and failure due to chronically enhanced catecholamine release (Hein, Altman, & Kobilka, 1999) and sympathectomy prevents cardiac fibrosis in hypertensive rats independent of blood pressure (Perlini et al., 2006). Also, mice that are unable to synthesize norepinephrine exhibit less cardiac hypertrophy and preserved ventricular function after aortic banding (Esposito et al., 2002). Overexpression of β1-adrenergic receptors in the heart produces a cardiomyopathic phenotype (Seeland et al., 2007). Prolonged *in vitro* exposure to norepinephrine or isoproterenol increase the number of apoptotic myocytes via stimulation of the β1-adrenergic receptor pathway (Communal, Singh, Pimentel, & Colucci, 1998; Iwai-Kanai et al., 1999), and

β1-adrenergic receptor selective antagonists completely prevent norepinephrine-stimulated apoptosis (Morisco, Zebrowski, Vatner, Vatner, & Sadoshima, 2001; Osadchii, 2007).

The β1-adrenergic receptor, coupled to Gs, exerts a pro-apoptotic action via a cAMP/PKA-dependent mechanism, which appears to involve mitochondria and ROS, and is associated with the activation of glycogen synthase kinase-3$\beta$ /JNK and p38 MAPK. Pro-apoptotic action of β1-adrenergic receptor may also involve activation of calcium/calmodulin-dependent protein kinase II (CaMKII). (Lamba & Abraham, 2000; Singh et al., 2001; Zaugg & Schaub, 2004). Amin et al. (2011) have shown that chronic β-adrenergic stimulation causes cardiomyocyte apoptosis and myocardial remodeling, and that the involvement of β1 integrin has protective effects via the FAK and PI3-kinase/Akt pathways (Figure 5) (Amin, Singh, & Singh, 2011).

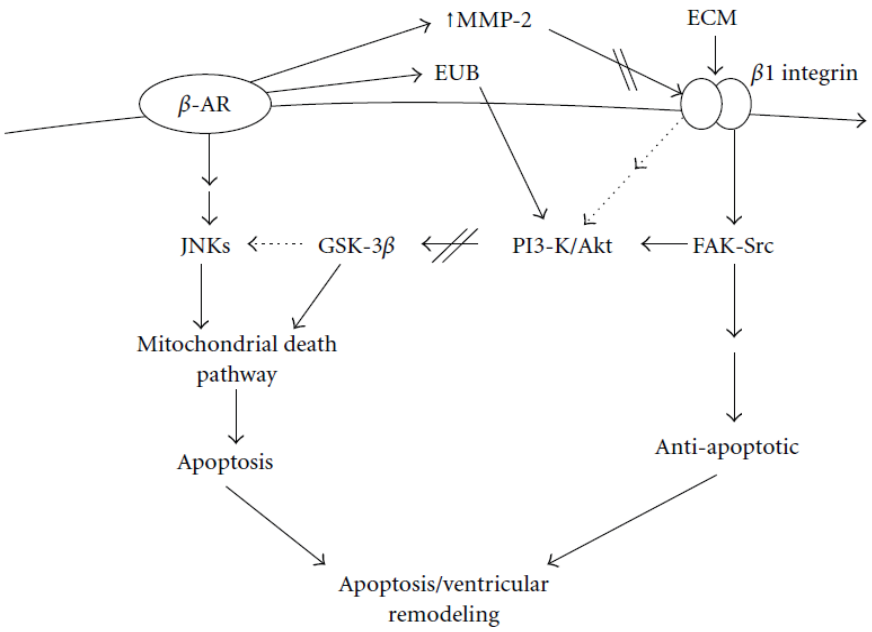


Figure 5: Schema illustrating the interaction between the β-adrenergic receptor and the β1 integrin signalling in cardiomyocytes. β-AR: Beta adrenergic receptor; ECM: extracellular matrix; MMP-2: Matrix metalloproteinase 2; EUB: Extracellular ubiquitin; PI3-K: phosphatidylinositol-3 kinase; FAK: Focal adhesion kinase; GSK: glycogen synthase kinase (Amin et al., 2011).

The β2-adrenergic receptor couples to Gs, Gi, or Gq depending on its functional status (Zaugg & Schaub, 2004). Similarly to β1-adrenergic receptor, β2-adrenergic receptor coupling to Gs exerts a pro-apoptotic action via cAMP pathways. Alternatively, β2-adrenergic coupling to Gi exerts an anti-apoptotic action which is mediated by PI3-kinase/Akt. Thus, in some cases, β2-adrenergic receptor activity in cardiomyocytes may oppose some of the deleterious effects of long-term β1-adrenergic receptor stimulation (Singh et al., 2001; Zaugg & Schaub, 2004).

The α1-adrenergic receptor signals through Gq/G11-Phospholypase C pathway, leading to an increase in intracellular calcium and an increase in contractility (Lamba & Abraham, 2000; Shannon & Chaudhry, 2006; Woodcock, 2007; Zaugg & Schaub, 2004). Overexpression or activation of α1-adrenergic receptors also results in cardiomyocyte hypertrophy and reduced apoptosis, through extracellular signal-regulated kinases (ERK) activation (Singh et al., 2001; Woodcock, 2007). So, depending on the balance between the activities of α1-, β2- and β1-adrenergic receptors, adrenergic activation can be pro- or anti-apoptotic for the cardiomyocyte (Figure 6).

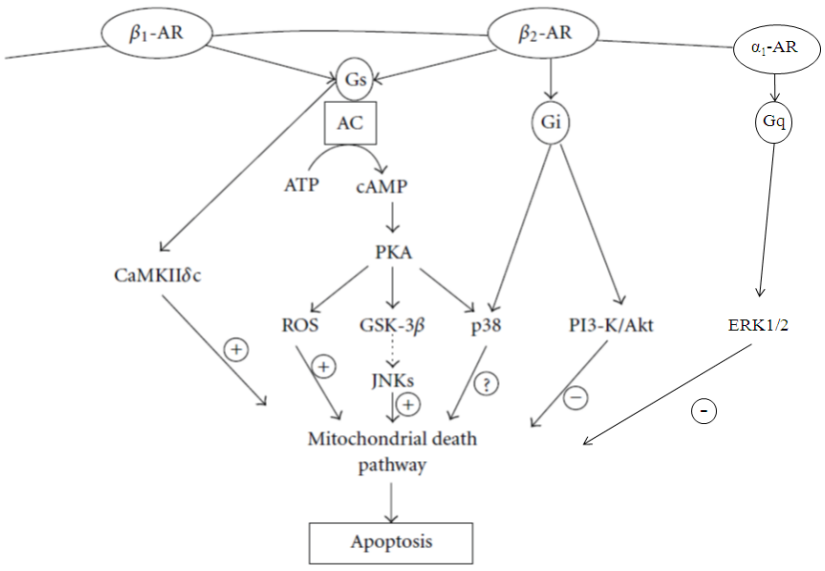


Figure 6: Signalling pathways involved in adrenergic receptor-mediated regulation of cardiomyocyte apoptosis. AR: adrenergic receptors; JNKs: c-Jun-N-terminal kinase; GSK-3β: glycogen synthase kinase-3β; ROS: reactive oxygen species; PKA: protein kinase A; AC: adenylyl cyclase; Gs: stimulatory G protein; Gi: inhibitory G protein; Gq: phospholipase C activating G protein; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; CaMKIIδ: calcium calmodulin kinase IIδ; PI-3K: phosphatidylinositol 3-kinase. Modified from Amin et al. (2011) and Sing et al. (2001) (Amin et al., 2011; Singh et al., 2001).

Chronic adrenergic stimulation of 1-week-old rat heart fibroblasts induces proliferation and stimulates collagen production (Bhambi & Eghbali, 1991; Lai, Sanderson, & Yu, 2009), through β1- and α1-adrenergic receptors, respectively (Lai et al., 2009). On the other hand, exposure of adult rat fibroblasts to norepinephrine causes cell proliferation, by a β2- but not β1- or α1-adrenergic receptor action (Leicht, Greipel, & Zimmer, 2000). This proliferative effect is mediated through ERK activation and production of interleukin (IL)-6, being decreased by blocking any one of these two mechanisms (Leicht, Briest, & Zimmer, 2003; Leicht et al., 2000). Intriguingly, β2-adrenergic receptor activation in adult rat fibroblasts results in reduced collagen I deposition, secondary to an increase in degradation. The importance of this effect in vivo remains to be demonstrated, but it is tempting to view this effect as a possible counterbalancing mechanism mediated by β2-adrenergic receptors (Aranguiz-Urroz et al., 2011).

In general, fibroblasts respond to noxious stimuli by morphologically and functionally changing to myofibroblasts, which are characterized by the expression of α-smooth muscle actin (Baum & Duffy, 2011; Rohr, 2011). These cells are metabolically active and contractile, have the ability to migrate, and locally produce inflammatory factors, including IL-1β, TNF-α and nitric oxide (NO) (Baum & Duffy, 2011; Turner et al., 2009). Noteworthy, fibroblasts in culture show markers of myofibroblast differentiation as early as the first passage (Rohr, 2011; Santiago et al., 2010), indicating that, in the literature, most of the in vitro studies on cardiac fibroblasts essentially refer to myofibroblasts (Baum & Duffy, 2011; Rohr, 2011).

Taken together, these studies demonstrate that excess catecholamines accelerate left ventricular remodelling and worsen myocardial function. The effects are mediated through direct activation of adrenergic receptors present in the heart. Furthermore, left ventricular remodelling and regulation of cardiac function may also be mediated indirectly, through catecholamine activation of the renin-angiotensin-aldosterone system, inflammatory cytokines, and oxidative stress.

### 1.4.2.2 The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) is considered a key factor in the long term regulation of blood pressure. The system is composed of a series of enzymatic cleaves that lead to the production of angiotensin II and aldosterone (reviewed by Carey, 2013 and Nguyen Dinh Cat and Touyz, 2011) (Carey, 2013; Nguyen Dinh Cat & Touyz, 2011). The classical RAAS is composed of the following cascade: renin, an enzyme that metabolizes plasma angiotensinogen into angiotensin I; then the angiotensin converting enzyme cleaves angiotensin I to produce the active vasoconstricting peptide, angiotensin II. The activity of angiotensin II in the suprarenal gland induces the production of aldosterone (Figure 7) (Carey, 2013; Kurtz, 2012; Nguyen Dinh Cat & Touyz, 2011).

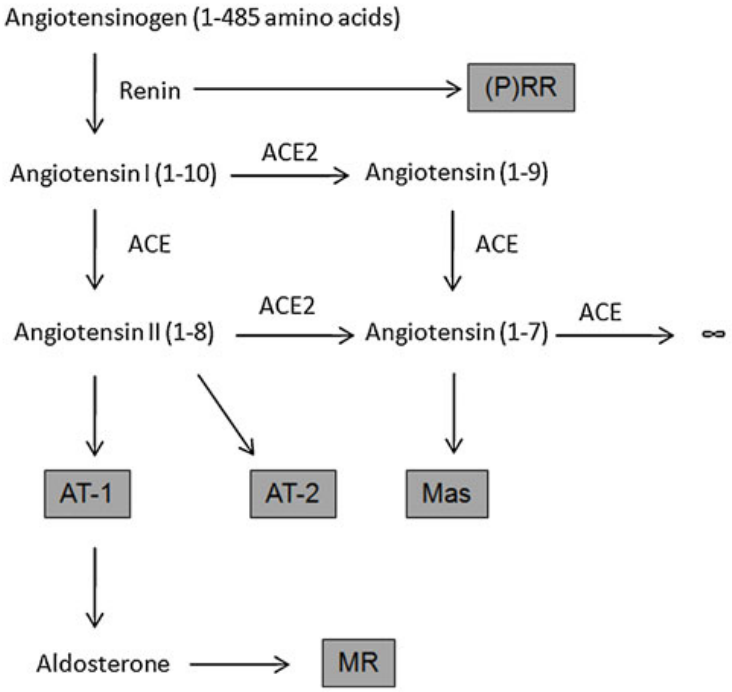


Figure 7: Current view of the renin-angiotensin-aldosterone system. ACE, angiotensin converting enzyme; (P)RR, (pro)renin receptor; AT-1 and AT-2, angiotensin receptor 1 and 2; Mas, Mas receptor; MR, mineralocorticoid receptor. Reviewed by Fournier et al., 2012 (Fournier, Luft, Bader, Ganten, & Andrade-Navarro, 2012).

Renin is produced in the kidney from cells located in the afferent arteriole, a zone known as the macula densa (De La Pena & De Castro, 1960). This anatomic localization allows for an easy detection of low blood pressure, one of the factors that control renin production (Blaine & Davis, 1971). Other mechanisms that stimulate renin release include reduced renal perfusion and high salt content in the distal tubules (Khayat, Gonda, Sen, & Smeby, 1981; Nguyen Dinh Cat & Touyz, 2011) as well as catecholamine activation of the β1-adrenergic receptor localized in the macula densa (Holmer et al., 1997; Weber, Brodde, Anlauf, & Bock, 1983). On the other hand, a negative feedback has been observed in the RAAS, since angiotensin II decreases renin secretion (Khayat et al., 1981).

The angiotensin II effects are mediated by angiotensin receptor 1 and 2 (AT1 and AT2 receptors). The AT1 receptor is considered the main effector of the RAAS, generating vasoconstriction, increasing renal sodium reabsorption, and inducing cardiomyocyte hypertrophy (Gray, Long, Kalinyak, Li, & Karliner, 1998) and apoptosis (Goldenberg, Grossman, Jacobson, Shneyvays, & Shainberg, 2001), with associated cardiac fibrosis (Lijnen, Petrov, & Fagard, 2000), and induces aldosterone production and secretion (Tanabe et al., 1998). Furthermore, elevated angiotensin II levels in the central nervous system induce sympathetic activity (Gao et al., 2005), creating a positive feedback loop in cardiovascular diseases (reviewed by Zucker et al. 2014) (Zucker, Xiao, & Haack, 2014).

In contrast, the AT2 receptor has vasodilatory (Siragy & Carey, 1997), natriuretic (Lo, Liu, Lantelme, & Sassard, 1995) and anti-fibrotic effects (Kurisu et al., 2003), without a direct effect on aldosterone secretion (Aguilera, 1992; Tanabe et al., 1998). The effect of AT2 receptor activity on cardiomyocyte hypertrophy is controversial, as studies have shown no change, decrease, or increase in hypertrophy following AT2 receptor activity (reviewed by Carey and Siragy 2003 and Carey 2013) (Carey, 2013; Carey & Siragy, 2003).

The final step in the classical RAAS system is the secretion of aldosterone from the adrenal medulla, following AT1 receptor activation (Aguilera, 1992; Shapiro et al., 2010). The main regulators of aldosterone secretion are changes in the extracellular potassium and the activity of angiotensin II (reviewed by Beuschlein 2013) (Beuschlein, 2013). Aldosterone increases renal sodium reabsorption (Fine, Meiselas, & Auerbach, 1958), increases blood pressure in nephrectomized animals (Friedman, Friedman, & Nakashima, 1958), with vasoconstriction,

endothelial dysfunction and later vascular remodelling (reviewed by Tomaschitz et al. 2010) (Tomaschitz, Pilz, Ritz, Obermayer-Pietsch, & Pieber, 2010), by action on the mineralocorticoid receptor (Pruthi et al., 2014). Additionally, aldosterone has been linked to cardiomyocyte hypertrophy (Rossier, Python, & Maturana, 2010), oxidative stress (Nolly et al., 2014), and cardiac fibrosis (Lin et al., 2012). Accordingly, the main effectors of the classical RAAS system increase blood pressure and have a deleterious cardiac effect, inducing hypertrophy and ventricular fibrosis.

In parallel, angiotensin1-7 is produced from Angiotensin I through 2 step-cleavage by ACE2 and ACE (Schiavone, Santos, Brosnihan, Khosla, & Ferrario, 1988), causing vasodilatation, inhibition of renal sodium reabsorption, and anti-fibrosis (Pinheiro et al., 2009; Santos, 2014), thus acting as a functional antagonist of the classical RAAS. Angiotensin1-7 vasodilatory actions are mediated by the MAS receptor (Peiro et al., 2013), a G-protein coupled receptor mediating all cellular effects of angiotensin1-7 (Santos et al., 2003).

#### 1.4.2.3 Inflammation and its effects on the development of LVH

Another mechanism involved in the changes following chronic cardiac overload is inflammation. Chronic, low level inflammation is part of the physiopathology of cardiac changes of both hypertension and heart failure (Ahn & Kim, 2012; Schiffrin, 2013; Wrigley, Lip, & Shantsila, 2011). Inflammation can induce cardiomyocyte apoptosis and fibrosis, on the other hand, it might also exert protective effects under some circumstances, for example in ischemia-reperfusion conditions (Ahn & Kim, 2012; Hedayat, Mahmoudi, Rose, & Rezaei, 2010).

The main inflammatory mediators are cytokines, paracrine factors that regulate immune responses (Gullestad et al., 2012). Cytokines are divided into interleukins, such as IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) and chemokines (Wrigley et al., 2011). TNF-α is mainly produced by cells of the immune system, especially from the monocyte/macrophage line (Kleinbongard, Heusch, & Schulz, 2010; Wrigley et al., 2011). In the heart, TNF-α is released by immune cells and also by both cardiomyocytes and fibroblasts in response to stress, such as that induced by catecholamines and oxidative stress (Hedayat et al., 2010;

Kleinbongard et al., 2010; Wrigley et al., 2011). Chronic norepinephrine stimulation of neonatal rat cardiomyocytes increases oxidative stress, which induces TNF-α synthesis and release (Fu et al., 2004).

IL-1β is produced by non-myocytes, primarily from macrophages that migrate to the myocardium following different inflammatory insults, and endothelial cells (Long, 2001). In fact, studies in vivo have shown a direct correlation between myocardial expression of IL-1β and myocardial invasion with monocytes, following experimental myocardial infarction or hypertension (Shioi et al., 1997; Yue, Massie, Simpson, & Long, 1998). In the same studies, no evidence of IL-1β secretion from cardiomyocytes was detected. Interestingly, in cultured cells, only fibroblast respond by secreting IL-1β in response to ischemic stress (Long, 2001; Porter & Turner, 2009), yet in pressure overload mice, IL-1β is produced in both cardiomyocyte and fibroblasts following stretch (Honsho et al., 2009). These results show the complexity of cardiac IL-1β regulation, but support the view that most of cardiac IL-1β secretion comes from non-myocyte sources.

Circulating and cardiac expression of TNF-α and IL-1β are increased in hypertension and heart failure, and have been implicated in LVH and remodeling (Aukrust et al., 1999; Duerrschmid et al., 2013; Gullestad et al., 2012; Timonen et al., 2008). Cytokines and other cytokine-related inflammatory factors contribute to cardiac remodeling by initiating or stimulating the cytokine cascade, inducing apoptosis/anti-apoptosis and regulating collagen (Hedayat et al., 2010; Kleinbongard et al., 2010; Siwik, Chang, & Colucci, 2000). Cytokines lead to cardiomyocyte hypertrophy and apoptosis *in vivo* and *in vitro* (Haudek, Taffet, Schneider, & Mann, 2007; Hiraoka et al., 2001; Hu et al., 2009), and stimulation of cardiac fibroblast proliferation and differentiation into activated myofibroblasts, which produce large amounts of collagens (Petrov, Fagard, & Lijnen, 2002). Cardiac over-expression of TNF-α results in cardiac fibrosis (Kubota et al., 2001; Zhang et al., 2011). Exposure to TNF-α reduces myocardial contractility, and, at long term, increases cardiomyocyte apoptosis and heart fibrosis (Hedayat et al., 2010). These long-term effects impede cardiac contractility and compromise cardiac performance (Haudek et al., 2007; Hiraoka et al., 2001; Sugden & Clerk, 1998). In fact, cytokines exert negative inotropic effects (Figure 8), thus aggravate already

impaired left ventricular function (Hedayat et al., 2010; Kubota et al., 2001; Yokoyama et al., 1993).

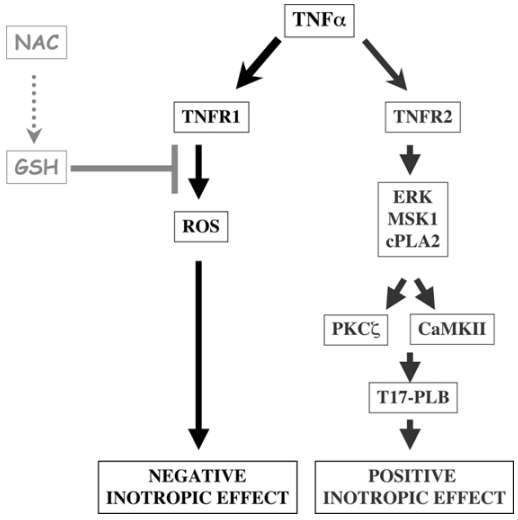


Figure 8: Opposing effects of TNFR1 and TNFR2 over cardiac contractility. Of note, the predominant effect in most of the cases is the TNFR1-mediated negative inotropic effect. TNFR: TNF-α receptor; MSK1: Mitogen- and stress-activated protein kinase-1; cPLA2: Cytoplasmic phospholipase A2; PKCζ: Protein kinase C ζ; CaMKII: Calcium calmodulin dependant protein kinase II; T17-PLB: Phospholipase B phosphorylated at threonine 17; ROS: Reactive oxygen species (Defer, Azroyan, Pecker, & Pavoine, 2007).

In the heart, the effects of IL-1β are controversial. IL-1β has been linked to the induction of apoptosis, ventricular remodelling, and cardiac dysfunction following acute non re-perfused infarct in mice (Toldo et al., 2013). In contrast, another study has shown that IL-1β knockout mice have increased ventricular apoptosis and fibrosis when subjected to aortic constriction, thus suggesting that IL-1β has a protective effect against pathological remodelling in this experimental model (Honsho et al., 2009). In pressure overload induced by aortic banding in mice, IL-1β is produced in both cardiomyocytes and fibroblasts, and results in ventricular hypertrophy, but maintains cardiac function, and modulates fibrosis (Honsho et al., 2009). The protective effect of IL-1β in overloaded ventricles includes the expression of insulin growth factor 1 (IGF-1), with downstream activation of ERK and Akt (Honsho et al., 2009). In contrast, the use of Anakinra, a recombinant form of the IL-1 receptor antagonist, in humans post-infraction reveals a tendency towards a decrease in development of heart failure (Abbate et al., 2013).

In addition, IL-1β reduces fibroblast proliferation, matrix activity, cell motility and α-smooth muscle actin expression, which are all hallmarks of myofibroblast differentiation (Bronnum, Eskildsen, Andersen, Schneider, & Sheikh, 2013). These findings suggest that IL-1β, besides its acknowledged adverse role in the inflammatory response, can also exert beneficial effects in cardiac fibrosis by actively suppressing differentiation of cardiac fibroblasts into fibrogenic myofibroblasts (Bronnum et al., 2013).

*In vitro*, cytokines have been shown to increase, decrease, or not change cardiomyocyte survival (Higuchi et al., 2002; Ing, Zang, Dzau, Webster, & Bishopric, 1999; Krown et al., 1996). These heterogeneous results depend on the experimental conditions as well as on the complex intracellular mechanisms which mediate cytokine actions. TNF-α and IL-1β primarily bind to respective receptors to modulate the activities of common intracellular signalling pathways involved in cell apoptosis or anti-apoptosis. These pathways include cytokine activation of MAPKs: ERK1/2, p38, and JNK (Bogoyevitch et al., 1996; Dhingra, Sharma, Singla, & Singal, 2007; Sugden & Clerk, 1998; Yndestad et al., 2010). The ERK1/2 cascade mediates cell growth and survival signals, while p38 and JNK are generally pro-apoptotic (Mizote et al., 2010; Yeh et al., 2010). The effects are achieved through regulation of gene and protein expressions, either through direct phosphorylation of transcription and translation factors, or via intermediate kinases (Mizote et al., 2010). Cytokines activate the transcription factor nuclear factor-κB (NF-κB), which in turn, is apoptotic and anti-apoptotic. NF-κB induces inducible nitric oxide synthase (iNOS) expression and NO production, which can contribute to cell death or block the apoptosis signalling cascade (Haudek et al., 2007; Hiraoka et al., 2001; Hu et al., 2009; Lu et al., 2008). In addition, TNF-α and IL-1β increase the intracellular production of second messenger sphingolipid metabolites: ceramide, sphingosine, and the phosphorylated derivative of sphingosine, sphingosine 1 phosphate (S1P) (Krown et al., 1996). Ceramide and sphingosine induce growth arrest and apoptosis, through protein kinase C (PKC) inhibition and downregulation of Akt phosphorylation and Bcl-2 (Hannun & Obeid, 2008). Conversely, S1P inhibits apoptosis and promotes proliferation through upregulation of Akt (Spiegel & Milstien, 2003). Thus, a dynamic balance between pro-apoptotic and anti-apoptotic pathways determines the fate of the cells whether to die or survive.

In fibroblasts, both proliferation, mediated by ERK and NF-κB (He et al., 2011) or apoptosis, mediated by NO and peroxynitrite (Tian, Liu, Bitterman, & Bache, 2002), have been described in vitro, again raising the possibility of a dual effect of IL-1β in cardiac cells.

In summary, pro-inflammatory cytokines IL-1β and TNF-α activate multiple signal transduction pathways inducing or suppressing a wide variety of genes, including those encoding for other cytokines and iNOS. It is important to note that the effects of cytokines, including left ventricular dysfunction may be completely reversed once cytokine production is either blocked or attenuated (Mann & Young, 1994; Remick et al., 2001). However, recent clinical trials of anti-TNF-α therapy in heart failure patients have been disappointing (Chung, Packer, Lo, Fasanmade, & Willerson, 2003; Mann et al., 2004). The failure of these treatments has been explained by the protective effect of TNF-α on cardiomyocytes under some physiological circumstances as well as lack of effect of these therapies on other pro-inflammatory cytokines (Gullestad et al., 2012).

#### 1.4.2.4 Natriuretic peptides as part of the cardiac response to stress

Natriuretic peptides are elevated in pathological states, specifically in hypertension associated with left ventricular hypertrophy (Irzmanski et al., 2007). In the adult mammalian heart, ANP is mainly produced by the cardiac atria, whereas ventricular synthesis of ANP and BNP is very low. However, ventricular peptide synthesis is re-induced when hypertrophy develops, so that ANP expression in the ventricles is frequently used as a marker for myocyte hypertrophy (Kaganovsky et al., 2001; Takemura et al., 1989).

It is important to note however, that elevated ANP and BNP in the heart may act as counter-regulatory mechanisms against further hypertrophy, heart failure, and its consequences (Mukaddam-Daher, 2006; Ogawa & de Bold, 2014). This is based on multiple studies showing that, independent of their role in blood pressure control, natriuretic peptides have direct anti-hypertrophic and anti-fibrotic actions on the heart. Deletion of a single copy of the pro-atrial natriuretic peptide gene (*Nppa*$^{+/-}$) results in exaggerated cardiac hypertrophy and remodeling after pressure overload stress (Franco et al., 2004). Mice lacking the natriuretic peptide receptor A (NPR-A) are hypertensive and display a marked cardiac (atrial and ventricular) hypertrophy,

with extensive fibrosis and chamber dilatation by 3 months of age, as well as high mortality rate due to sudden death (Lopez et al., 1995). Although BNP and ANP signal through the same NPR-A receptor, mice with disrupted BNP exhibit a different phenotype than ANP-deficient mice. BNP knockout mice are not hypertensive and do not develop ventricular hypertrophy, but develop multifocal fibrotic lesions in their ventricles (Tamura et al., 2000). Also, ANP and BNP suppress hypoxia-induced collagen synthesis in fibroblasts (Tamamori, Ito, Hiroe, Marumo, & Hata, 1997).

The anti-growth actions of natriuretic peptides are mediated by different mechanisms. Natriuretic peptides inhibit MAPK via the inhibition of upstream kinase(s), including MEK, uniquely resulting from ligand binding to NPR-C clearance receptor (Prins et al., 1996). Also, ANP/NPR-A system negatively regulates MAPK/ERK2 activity and proliferation of mesangial cells in a PKG-dependent manner (Pandey, Nguyen, Li, & Boyle, 2000).

Kato et al. (2005) reported that high concentrations ($10^{-6}$ M) of ANP stimulate apoptosis, evidenced by increased caspase 3, but at lower concentrations, ANP was anti-apoptotic, protecting cardiomyocytes from staurosporine challenge, by mechanisms that include cGMP-dependent nuclear accumulation of Akt (Kato et al., 2005). The anti-apoptotic effect of natriuretic peptides was also shown in serum-deprived PC12 cells, where prolonged cGMP elevations caused by ANP or BNP inhibited apoptotic DNA fragmentation and prolonged cell survival (Fiscus, Tu, & Chew, 2001). The protective effect may involve suppression of cytokine production, as ANP attenuates TNF-$\alpha$ production by macrophages, by suppressing p38 MAPK and NF-$\kappa$B activation (Tsukagoshi et al., 2001) and protects from ischemia-reperfusion liver injury by reducing TNF-$\alpha$ mRNA expression and NF-$\kappa$B activation via cGMP (Kiemer, Vollmar, Bilzer, Gerwig, & Gerbes, 2000).

Furthermore, the natriuretic peptide interaction with nitric oxide (NO) places ANP in a unique position of being able to maintain the essential or protective actions of NO while inhibiting potentially cytotoxic or detrimental effects (Drexler, 1999). ANP stimulates endothelial nitric oxide synthase (eNOS) (Murthy, Teng, Jin, & Makhlouf, 1998), but inhibits pro-inflammatory cytokine-induced nitric oxide (iNOS) production in human proximal tubular cells in culture exposed to a combination of IL-1$\beta$, TNF-$\alpha$, and IFN-$\gamma$ (Chatterjee, Hawksworth, & McLay,

1999). In contrast, IL-1β-stimulated iNOS mRNA and nitric oxide production from ventricular cardiomyocytes (Harding, Carretero, & LaPointe, 1995) and cultured rat vascular smooth muscle cells (Iimura et al., 1998) is augmented in the presence of ANP and BNP (Yamamoto, Ikeda, & Shimada, 1997). These studies imply cell and stimulus-dependency.

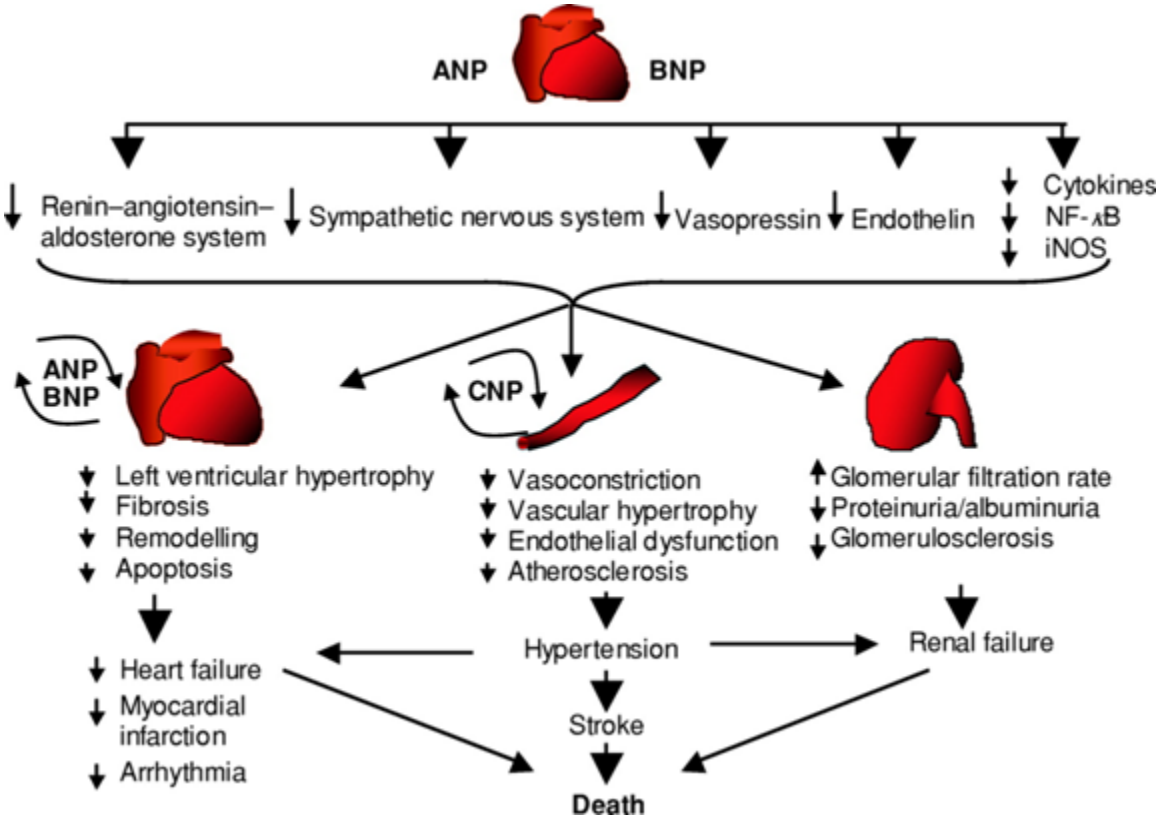


Figure 9 : Protective effects of natriuretic peptides in cardiac pathology (reviewed by Mukaddam-Daher, 2006) (Mukaddam-Daher, 2006).

In summary, natriuretic peptides serve as acute and chronic control mechanisms against cardiac overload, serving as a counterbalancing mechanism, reducing cardiac remodelling. Through sympatholytic and anti-inflammatory actions, natriuretic peptides are anti-hypertrophic, anti-proliferative and anti-fibrotic (Ogawa & de Bold, 2014). Based on their multiple protective effects (Figure 9) pharmacological therapy has been approved and includes inhibition of endogenous natriuretic peptide degradation or by potentiating natriuretic peptide

levels by intravenous infusion of recombinant ANP or pro-ANP (reviewed by: Mukaddam-Daher, 2006; George et al., 2014) (George, Rajaram, Shanmugam, & Vijayakumar, 2014; Mukaddam-Daher, 2006). Other pharmacological compounds that potentiate endogenous natriuretic peptides include β-blockers (atenolol, carvedilol, propranolol, bisoprolol, tertatolol) and centrally-acting sympatholytic drugs, like clonidine and, recently, moxonidine (El-Ayoubi, Menaouar, Gutkowska, & Mukaddam-Daher, 2005; Mukaddam-Daher & Gutkowska, 1999, 2000). Clinically, moxonidine has been shown to be a very efficacious antihypertensive agent with cardioprotective effects that, at least in part (as presented below, section 1.6.1 Moxonidine in hypertension), may be mediated by potentiated natriuretic peptide levels.

## 1.5 Current pharmacological treatment options for hypertension and heart failure

Current heart failure treatment guidelines emphasizes treatment of predisposing factors as a first step of treatment , and thus overlap with treatment guidelines for other cardiovascular pathologies, especially hypertension, which is a major risk factor for heart failure (Hunt et al., 2009; Kannel et al., 1972).

Hypertension treatment recommendations include changing lifestyle, such as weight loss, exercise, and reduction of sodium intake and stress levels. These treatment venues can be moderately effective (for example reducing salt intake can decrease mean blood pressure around 4 mmHg) (Chobanian et al., 2003; Mancia et al., 2007), yet most hypertensive patients need pharmacological treatment, and in most cases 2 or more drugs are required (Chobanian et al., 2003; Hackam et al., 2013; James et al., 2014).

Several classes of antihypertensive medications are currently available, including vasodilators, thiazide diuretics, RAAS blockers, including angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB), long acting calcium channel antagonists, and sympatholytics, including β-adrenergic blockers and centrally acting α2-adrenergic and imidazoline $I_1$-receptor agonists (Brunton et al., 2011; Hackam et al., 2013; Mancia et al., 2007; Poirier & Lacourciere, 2012). The choice of therapy depends on the presence of other

risk factors, such as LVH, diabetes, obesity, and others. It is important that in addition to lowering blood pressure, pharmacological antihypertensive therapy takes into consideration these risk factors, thus should aim at regressing LVH, improving cardiac function, as well as improving, or at least not worsening, insulin sensitivity and lipid metabolism (Hackam et al., 2013; James et al., 2014).

In this respect, some medications, while excellent vasodilators, reducing blood pressure, are not part of the current treatment of hypertension due to their lack of effect on complications, like LVH, or even an increase in the risk of cardiovascular events due to their ability to activate the sympathetic nervous system (Brunton et al., 2011). Hydralazine is a potent arterial vasodilator, yet it increases sympathetic activity and does not regress LVH (El-Ayoubi et al., 2004; Leenen, Burns, Myers, & Frankel, 1990; Tsoporis & Leenen, 1988). Thiazide diuretics also induce dyslipoproteinemia and stimulate the sympathetic nervous system (Pischon & Sharma, 2002). Calcium channel blockers are considered metabolically neutral and are successfully used to treat patients with hypertension and stable angina pectoris, however they may have unfavorable effects in patients with impaired left ventricular function, due in part to baroreceptor-mediated activation of the SNS (Toal, Meredith, & Elliott, 2012), an effect which could decrease their benefit (Fisher & Fadel, 2010; Toal et al., 2012).

On the other hand, inhibitors of the RAAS are very effective antihypertensive treatments, improving cardiac function and having a favorable metabolic profile, producing increases in insulin sensitivity (Manrique, Lastra, Gardner, & Sowers, 2009). In addition to opposing/inhibiting the deleterious effects of RAAS, the important clinical benefits of ACE inhibitors and angiotensin II receptor blockers (ARBs) could also be explained by their blunting or inhibiting the SNS effects in patients with cardiovascular disease (De Tommasi et al., 2003); reviewed by Nap et al. 2003 (Nap, Balt, Mathy, & Van Zwieten, 2003).

Therefore, it appears imperative that selection of vasoactive drugs for the treatment of cardiovascular diseases take into consideration their effects on the sympathetic nervous system, where, ideally, treatment should also blunt sympathetic activity, or at least avoid activating it.

It is evident that sympathetic nervous system dysfunction, manifested as persistent and adverse activation of sympathetic outflows to the heart and kidneys, is crucial in the development of

heart failure and hypertension (Grassi, 2013; Grassi et al., 2010). Current strategies that directly target the activity of the sympathetic nervous system include β-adrenergic receptor blockers and centrally acting medications. Both groups attenuate sympathetic effect on target organs (Brunton et al., 2011; Fisher & Fadel, 2010). In fact, these medications, although routinely used in treatment of hypertension (Hackam et al., 2013; Mancia et al., 2007), remain underutilized. The use of some β-blockers is waning because of their metabolic effects, including weight gain and predisposition to diabetes development (Brunton et al., 2011). However, in patients with reduced ejection fraction, or symptomatic heart failure, modulation of the activity of the sympathetic nervous system with the use of β-blockers is an integral part of treatment (Hunt et al., 2009). In heart failure patients, β-blockers increase ejection fraction (Gilbert et al., 1996), increase exercise capacity (Dekleva et al., 2012), and reduce heart failure symptoms and mortality (DiNicolantonio, Lavie, Fares, Menezes, & O'Keefe, 2013; Hunt et al., 2009). It is worth noting that during β-blockade, adverse effects of norepinephrine may be observed following unopposed α1-adrenergic stimulation. In this respect, the combined β- and α1-adrenergic blocker carvedilol is favored as it improves cardiac output and mortality in patients with heart failure (Brodde & Michel, 1999; Packer et al., 1996). These results stress the importance of correctly modulating sympathetic nervous system activity in heart failure and favor inhibition of norepinephrine release rather than actions. Centrally acting drugs, such as clonidine and α-methyldopa, reduce central sympathetic drive and norepinephrine release. These drugs, consequently, maintain cardiac output, arterial tone, and body fluid volume (Azevedo, Newton, & Parker, 1999; Brunton et al., 2011; Sanjuliani, Francischetti, Genelhu de Abreu, & Ueleres Braga, 2004). Despite their clinical efficacy, the use of these "first generation centrally acting drugs" was often limited by their side effects, which include dry mouth, sedation, and mental depression (Brunton et al., 2011; Webster & Koch, 1996). Also, rebound over-activity of the sympathetic nervous system has been described following sudden discontinuation of clonidine (Brunton et al., 2011; Webster & Koch, 1996). However, the discovery of imidazoline $I_1$-receptors and development of selective compounds, moxonidine and rilmenidine, which show an improved side-effect profile (in comparison to clonidine), revived interest in centrally acting drugs (Edwards, Brown-Bryan, McLean, & Ernsberger, 2012). The improved side-effect profile is based on the higher affinity of moxonidine and rilmenidine to

imidazoline $I_1$-receptors and lower affinity to α2-adrenergic receptors (Ernsberger, Giuliano, Willette, Granata, & Reis, 1988).

## 1.6 Moxonidine

Moxonidine (4-chloro-N-(4,5-dihydro-1H-imidazol-2-yl)-6-methoxy-2-methyl-5-pyrimidinamine), the prototype of the "second generation centrally acting drugs", is a chemical derivate of clonidine that retains the 5-membered imidazoline ring, but has different substitutions in other parts of the molecule (Figure 10) (Ernsberger, 2000). Moxonidine shows 70 times higher affinity to imidazoline $I_1$-receptors compared to α2-adrenergic receptors, as observed in binding assays performed in bovine rostro-ventro lateral medulla (RVLM) (Ernsberger, Damon, Graff, Schafer, & Christen, 1993). Thus, moxonidine is an effective antihypertensive compound, with far less side effects, and no rebound effect upon discontinuation (Planitz, 1987), most likely due to its low affinity for the α2-adrenergic receptor (Ernsberger, 2000; Ernsberger et al., 1993).

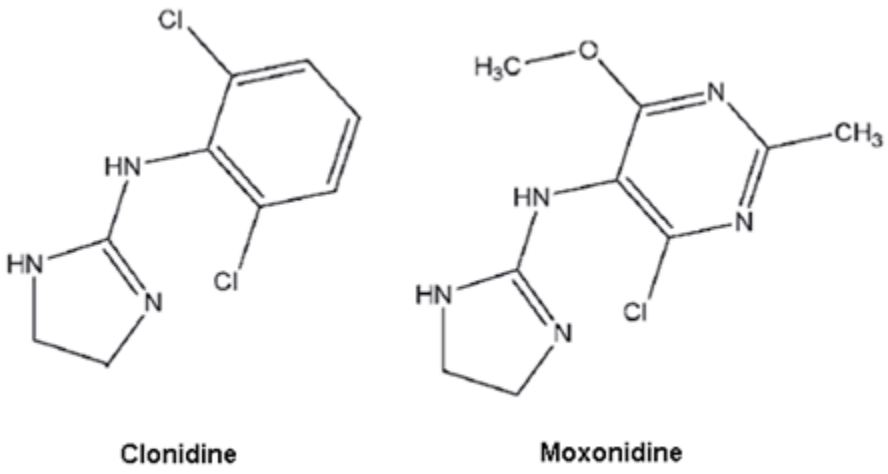


Figure 10 : Structure of imidazoline agonists clonidine and moxonidine.

### 1.6.1 Moxonidine in hypertension

Moxonidine is an antihypertensive medication with an oral half-life of 2.1 hours (Trenk, Wagner, Jahnchen, & Planitz, 1987), but approved to be used once a day, due to its lasting actions on blood pressure (Edwards et al., 2012). In humans, moxonidine reduces blood pressure, with good tolerability (Prichard et al., 2002). Its blood pressure-lowering effect equals that of thiazides, calcium antagonists, ACE inhibitors, α- and β-adrenergic receptor blockers, and the first generation centrally-acting antihypertensives, such as α-methyldopa and clonidine (Fenton, Keating, & Lyseng-Williamson, 2006; Kuppers et al., 1997; Ollivier & Christen, 1994).

In addition to blood pressure reduction, moxonidine improves glycemic control in patients with diabetes mellitus or metabolic syndrome (Chazova & Schlaich, 2013; Derosa et al., 2007; Kemme et al., 2003) and reduces bodyweight in hypertensive obese patients (Sharma, Wagner, & Marsalek, 2004). Also, moxonidine reduces plasma TNF-α level in hypertensive and overweight postmenopausal women (Poyhonen-Alho et al., 2008). Taken together, these studies support the use of moxonidine in patients with hypertension complicated with metabolic syndrome.

Moxonidine results in a clinically relevant reduction in blood pressure in patients with renal disease and resistant hypertension; and may be an "add-on" treatment in elderly patients whose hypertension is poorly controlled despite treatment with two or more antihypertensive agents (Hausberg, Tokmak, Pavenstadt, Kramer, & Rump, 2010; Martin, Hill, & O'Mahony, 2005; Neumann, Ligtenberg, Oey, Koomans, & Blankestijn, 2004). Furthermore, moxonidine has anxiolytic and anti-depressant effects, which may be especially important in cardiovascular diseases (Konstam, Moser, & De Jong, 2005).

Moxonidine treatment also regresses LVH in hypertensive patients and animals (Haczynski, Flasinski, Przewlocka-Kosmala, & Spring, 2001; Mall et al., 1991; Paquette et al., 2008). The anti-hypertrophic effect of moxonidine may include induction of natriuretic peptides, as suggested by studies performed in our laboratory on normotensive and hypertensive rats. Acute (1-4 h) (Mukaddam-Daher & Gutkowska, 1999, 2000) and chronic (1week) *in vivo* moxonidine treatment increase circulating ANP levels (Menaouar, El-Ayoubi, Jankowski,

Gutkowska, & Mukaddam-Daher, 2002). Furthermore, moxonidine stimulates ANP release from isolated rat hearts *ex vivo*, an effect mediated, in part, by cardiac imidazoline $I_1$-receptors (Mukaddam-Daher et al., 2006).

Previous observations from this laboratory have also shown that chronic treatment of spontaneously hypertensive rats (SHR) with non-hypotensive and hypotensive doses of moxonidine results in reduced left ventricular hypertrophy, by mechanisms that include a sustained decrease in DNA synthesis as well as by a transient increase in ventricular apoptosis (detected as an increase in DNA laddering and caspase 3 expression), appearing after 1 week and subsiding by 1 month of treatment (Paquette et al., 2008). However, based on the link between cardiomyocyte apoptosis and heart failure (Kemp & Conte, 2012), the effect of the transient apoptotic action of moxonidine in hypertension on cardiac function warrants further investigation.

### 1.6.2 Moxonidine in heart failure

Despite the overwhelming evidence that moxonidine is a safe and effective antihypertensive drug, its use in heart failure is controversial. Dickstein et al. (1999) reported that acute use of moxonidine in patients with heart failure markedly decreased plasma norepinephrine, implying a decrease in sympathetic output (Dickstein et al., 1999). This effect was later confirmed in a small 12-week trial, in which heart failure patients received moxonidine with good tolerability (Swedberg, Bergh, Dickstein, McNay, & Steinberg, 2000). In 2002, a study using different doses of moxonidine (extended release preparation) revealed that high doses of moxonidine were associated with a decrease in plasma norepinephrine, but an increase in adverse effects was also observed (Swedberg et al., 2002). In 2003, a phase III trial, the Moxonidine Congestive Heart Failure (MOXCON) trial was conducted on patients with NYHA class II-IV heart failure and LVEF $\leq$ 35%. This trial reported more deaths in the moxonidine-treated group (54 vs. 32 patients), which led to its abrupt termination without further investigation of the underlying mechanisms (Cohn et al., 2003). Several reasons may underlie the findings of the failed study, including very high doses (5 times higher than the recommended antihypertensive doses) leading to strong suppression of much needed residual

sympathetic activity to the heart, lack of patient compliance leading to rebound effect of the drug, increased myocardial metabolism, or interference with other heart failure medications (Coats, 1999; Floras, 2002; Mobini et al., 2006).

On the other hand, in post-infarction rats, a model of acute heart failure, moxonidine treatment reduced LVH and increased ventricular capillary density, implying an increase in myocardial oxygen supply to the cardiomyocytes (Van Kerckhoven, van Veghel, Saxena, & Schoemaker, 2004), a beneficial effect in post-infarcted hearts.

These studies clearly demonstrate that the mechanism of action of moxonidine may have alternative drug targets (adrenergic, non-adrenergic) or different target distribution (organ- or cell-specific), regulation, cell-specific signaling pathways, and interaction with other mechanisms stimulated in hypertension or heart failure.

## 1.7 The imidazoline $I_1$-receptor

Blood pressure reduction by the centrally acting compound moxonidine is mediated by 2 functionally and genetically distinct receptors: α2-adrenergic receptors and non-adrenergic imidazoline $I_1$-receptors, with higher affinity at imidazoline $I_1$-receptors (Ernsberger et al., 1993; Ernsberger et al., 1988).

### 1.7.1 Characterization of the imidazoline $I_1$-receptor

The existence of imidazoline receptors was first suggested by Bosquet and co-workers in 1984, following an experiment in which microinjections of the imidazoline compounds, cirazoline and ST-587 (both α1 adrenergic agonists), in cat's nucleus reticularis lateralis had hypotensive and bradicardic effects similar to clonidine, an imidazoline compound described at that time as a central α2-adrenergic agonist (Bousquet et al., 1984). On the other hand, injection of the catecholamine α-methylnorephinephrine, a putative α2-adrenergic agonist, injected in the same zone did not change heart rate or blood pressure (Bousquet et al., 1984). The investigators observed that only substances with an imidazoline 5-membered ring structure were hypotensive, which led them to suggest the existence of a site that binds

imidazoline compounds, but not catecholamines. Later, the existence of central "imidazoline preferring, non-α adrenergic membrane receptors" was confirmed by binding assays (Bricca et al., 1989).

Imidazoline receptors are a non-adrenergic heterogeneous group of proteins, broadly divided into $I_1$, $I_2$, and $I_3$, based on physiological function, location, and pharmacological tools available to differentiate them (Hamilton, 1995). Imidazoline $I_2$-receptors, mainly found in the brain, are involved in mood disorders (Garcia-Sevilla et al., 1995; Halaris & Piletz, 2003), while imidazoline $I_3$-receptors in the pancreas control insulin secretion (Morgan & Chan, 2001). On the other hand, imidazoline $I_1$-receptors (the focus of our studies) occur mainly in the brainstem medulla, adrenal chromaffin cells, and, to a lower extent, in the kidneys. (Vauquelin, De Backer, Ladure, & Flamez, 1999).

Our laboratory was the first to identify functional imidazoline $I_1$-receptors in the heart (Mukaddam-Daher et al., 1997). Using binding assays, Western blots, and immunohistochemical techniques, imidazoline $I_1$-receptors were localized to heart atria and ventricles of several species, including rats, hamsters, and humans (El-Ayoubi et al., 2002). Furthermore, imidazoline $I_1$-receptors were shown to be up-regulated in atria of SHR with already developed LVH, and in ventricles of hamsters with advanced cardiomyopathy, and in human ventricles with heart failure (El-Ayoubi et al., 2002). Heart imidazoline $I_1$-receptors, but not α2-adrenergic receptors, were altered by chronic *in vivo* treatment of hypertensive rats with moxonidine (El-Ayoubi et al., 2003, 2004). These studies indicated that heart imidazoline $I_1$-receptors are responsive to chronic exposure to agonist as well as to changes in the cardiovascular environment.

The nature of imidazoline $I_1$-receptor was not identified until in 1998, when the group of Piletz and Ernsberger characterized an 85 kilodalton (kDa) protein as a candidate for the imidazoline $I_1$-receptor in rat brain and in rat neuronal pheochromocytoma PC12 cells (Ivanov, Zhu, et al., 1998). The characterization of the receptor took a great leap forward once this group found that the imidazoline $I_1$-receptor was coded by the "imidazoline receptor antisera selected" (IRAS) cDNA, isolated from a library of human hippocampus cDNA, which was expressed in E. coli and tested for immunoreactivity with 2 specific anti-imidazoline binding protein antisera. The positive clones were sequenced and found to code for a single cDNA (Ivanov,

Jones, Dontenwill, Bousquet, & Piletz, 1998; Piletz, Jones, Zhu, Bishara, & Ernsberger, 1999). Transfection of IRAS full cDNA into Chinese hamster ovary cells (CHO) developed high-affinity imidazoline $I_1$-binding sites (Piletz et al., 2000).

Independently, Alahari and co-workers (Alahari et al., 2000), using a yeast two-hybrid system with the α5 integrin cytoplasmic tail as bait, and an embryonic mouse cDNA library coding for the prey proteins, discovered a ~190 kDa protein that binds to the integrin α5 tail of the α5β1 fibronectin receptor. Transfection of 3T3 embryonic fibroblast cell line or Chinese hamster ovary cells with cDNA coding for this protein slowed cell migration. Accordingly, Alahari called it Nischarin, a Sanskrit word that denotes slow motion (Alahari et al., 2000). Nischarin was further described as a cytosolic protein whose mRNA is expressed in several mouse tissues, including brain, kidney, lung, and heart. Sequencing of nischarin revealed 80% homology with human IRAS (Alahari et al., 2000), thus nischarin is the murine homologue of human IRAS. In PC12 cells, the use of IRAS/nischarin antisense lowers the density of imidazoline $I_1$-sites in the cells, providing evidence that nischarin expression is related to the imidazoline $I_1$-receptor (Sun, Chang, & Ernsberger, 2007). In addition, IRAS/nischarin antisense inhibited ERK activation induced by the selective imidazoline $I_1$-receptor agonists, rilmenidine (Zhang & Abdel-Rahman, 2006) or moxonidine (Sun et al., 2007), confirming the relation between nischarin expression and the imidazoline $I_1$-receptor.

Nischarin is located both in cell membrane and cytosol. In nischarin-transfected COS-7 fibroblast-like cells and non-transfected neuro 2A neuroblast cells, using sub-cellular fractionation by ultra-centrifugation, followed by Western blot, Alahari found that nischarin was mostly a cytosolic protein, with a small pool near the membrane that binds to the fibronectin receptor (Alahari et al., 2000). On the other hand, using immunofluorescence, Zhang and Abdel-Rahman (2006) found that PC12 pheochromocytoma cells express nischarin mainly in the cellular membrane (Zhang & Abdel-Rahman, 2006). Accordingly, nischarin localization may vary depending on the cell type studied.

In 2008, the group of Bousquet proposed that the imidazoline $I_1$-receptor functionally interacts with the G-protein inwardly rectifying potassium channel (GIRK). This hypothesis was based on intracisternal injections studies in rabbits, showing that opening the GIRK pore increased the hypotensive effects of the selective imidazoline $I_1$-receptor ligands LNP509 and LNP640,

and closing the pore diminished their actions (Yoro Sy et al., 2008). Also, binding studies performed on PC12 cells, showed that the binding of the selective imidazoline $I_1$-receptor ligand LNP911 was modified by substances that open or close the GIRK pore, without changing the affinity, suggesting that the imidazoline binding site could be located inside GIRK (Yoro Sy et al., 2008).

Finally, the group of Molderings, also using PC12 cells, and siRNA for the lysophospholipid receptors of S1P ($S1P_{1-3}$) and lysophosphatidic acid receptor families, have shown that binding of clonidine was markedly reduced by the inhibition of $S1P_1$ and $S1P_3$ expression, suggesting that the imidazoline $I_1$-receptor in PC12 cells is part of the lysophosholipid S1P receptor family ($S1P_1$ and $S1P_3$) (Molderings et al., 2007), and that it requires nischarin expression for its trafficking towards the membrane, thus nischarin is part of its signalling pathway (Molderings et al., 2007; Zhang & Abdel-Rahman, 2006).

Based on the above described studies, the imidazoline $I_1$-receptor/nischarin might interact with several membrane components. The importance of these interactions is not completely understood, but, it is clear that imidazoline $I_1$-receptor/nischarin expression is a key factor in the regulation and function of imidazolines.

## 1.7.2 Structure of imidazoline $I_1$-receptor/nischarin

Mouse nischarin is described as a 1593 amino acid (aa) protein (accession no NP_073147) (Lim & Hong, 2004), with multiple functional domains.

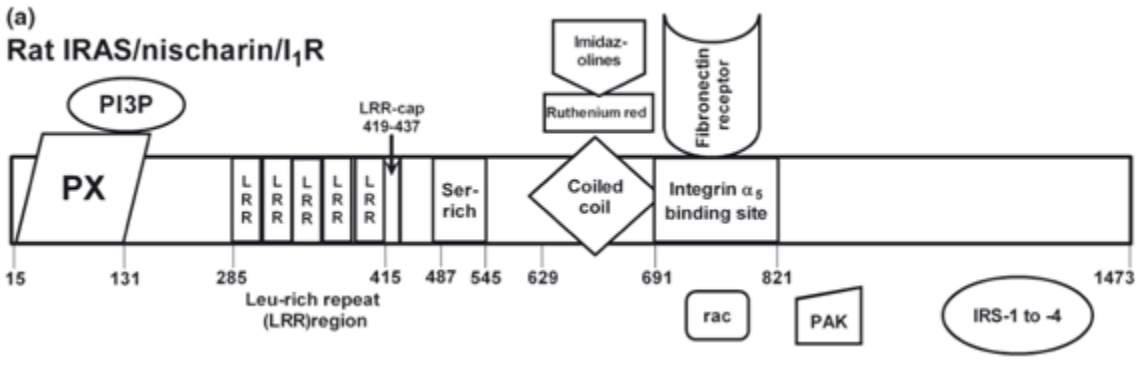


Figure 11 : Domain map of nischarin. For details see text (Sun et al., 2007).

Figure 11 shows the described domains of nischarin as compiled by Sun et al. (2007) (Sun et al., 2007). The PX domain describes a sequence that is common to a group of proteins called sorting nexins; this domain is necessary for nischarin targeting towards the cell membrane and trafficking towards endosomes (Lim & Hong, 2004). In addition, trafficking needs the presence of the coiled coil region, which allows nischarin to homo-oligomerize (Lim & Hong, 2004); the coiled coil region also binds ruthenium red, and has been proposed as the imidazoline binding site (Piletz et al., 2000; Sun et al., 2007). Leucine-rich repeats (LRR in Figure 11) have been described as sites for protein to protein interaction, with activities such as receptor regulation, cytoskeletal configuration and apoptosis regulation (reviewed by Kobe and Kajava, 2001) (Kobe & Kajava, 2001). The interaction partners for the 5 observed leucine-rich repeats sequences present in nischarin are currently unknown. Recently, the group of Alahari has described an interaction between nischarin and the tumor suppressor protein liver kinase B1 (LKB1), enhancing the kinase activity of this protein. This interaction enhances the tumor suppressor abilities of both nischarin and LKB1. The interaction site was mapped near the N-terminal site, between amino acids 416 and 624 (Jain et al., 2013).

Finally, there is the α5 integrin subunit binding site (part of the fibronectin receptor), described by Alahari and coworkers in the original publication describing nischarin (Alahari et al., 2000). Interestingly, overexpression of IRAS can redistribute surface α5 integrin to intracellular endosomal structures, and this could be linked to the observed alterations in migration following nischarin expression (Lim & Hong, 2004).

### 1.7.3 Signalling through the imidazoline $I_1$-receptor/nischarin

Due to the lack of specific imidazoline $I_1$-receptor agonists devoid of activity at the α2-adrenergic receptors, most of the imidazoline $I_1$-receptor signalling studies have been performed on neuronal rat pheochromocytoma cell line (PC12). These cells express imidazoline $I_1$-receptor but not α2-adrenergic receptor or $I_2$-receptors, as confirmed by RT-PCR and membrane binding studies (Molderings, Bonisch, Hammermann, Gothert, & Bruss, 2002).

In PC12 cells, activation of imidazoline $I_1$-receptors by moxonidine (Figure 12) activates phosphatidylcholine-selective phospholipase C (PC-PLC) and induces the accumulation of diacylglycerol (DAG) (Ernsberger, 1999; Separovic, Kester, & Ernsberger, 1996; Separovic, Kester, Haxhiu, & Ernsberger, 1997), and downstream activation of 2 isoforms of protein kinase C (PKC), specifically the $cPKC\beta_{II}$ and $aPKC_{\zeta}$ isoforms (Edwards et al., 2001). The activity of PKC induces the activation of ERK and JNK, but in the presence of nerve growth factor (NGF), a potent activator of neuronal PC12 cell differentiation, moxonidine almost completely reverses NGF-induced ERK activation (Edwards et al., 2001). Therefore, depending on the stimulus, imidazoline $I_1$-receptors can either activate or inactivate ERK.

Imidazoline $I_1$-receptor activation induces the production of arachidonic acid, which in turn produces prostaglandin E2 (PGE2), by mechanisms that include activation of PC-PLC and are independent of calcium or phospholipase $A_2$ (Ernsberger, 1998) (Figure 12). Interestingly, in the H9C2 cardiomyoblast cell line, blocking PGE2 production by the use of the cyclooxygenase 2 blocker celecoxif induces cell death (Sakane et al., 2014). These studies suggest a protective effect of locally produced PGE2 following imidazoline $I_1$-receptor activation.

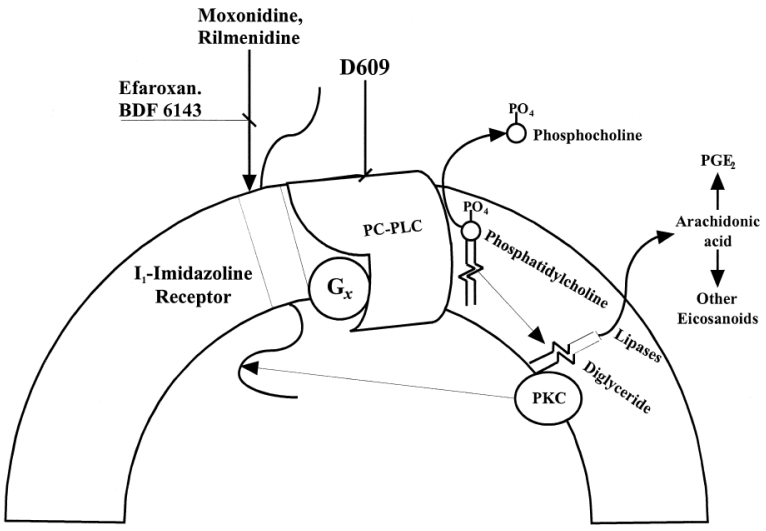


Figure 12 : Imidazoline $I_1$-receptor mediated production of PGE2 in PC12 cells (Ernsberger, 1998).

In vivo studies have shown that injecting the PC-PLC inhibitor D609 in the rostro-ventro-lateral medulla (RVLM) blocks the antihypertensive effect of moxonidine in hypertensive rats, and thus this mechanism is important in the physiological effects of the imidazoline $I_1$-receptor (Separovic et al., 1997). Furthermore, central blocking (using intracisternal cannulation) of ERK phosphorylation in the RVLM in Sprague-Dawley (SD) rats blocks the hypotensive effects of the imidazoline $I_1$-receptor agonist rilmenidine (Zhang & Abdel-Rahman, 2005). Additionally, the effect of rilmenidine on blood pressure reduction and ERK activation depends on the expression of nischarin, since blocking its expression in vivo by nischarin antisense reduces hypotension and ERK activation (Zhang & Abdel-Rahman, 2008), linking the ERK activation by imidazoline $I_1$-receptor agonists to the expression of nischarin. Finally, blocking either ERK or p38 MAPK activity in the central nervous system of rats with acute renal failure blocks the hypotensive effects of moxonidine, suggesting that, in vivo, the hypotensive effects of imidazoline $I_1$-receptor activation are mediated by both ERK and p38 MAPK (El-Mas, El-Gowelli, Ghazal, Harraz, & Mohy El-Din, 2009). These results imply that the imidazoline $I_1$-receptor/PC-PLC/ERK/p38 MAPK pathway mediates the central hypotensive effects of the imidazoline $I_1$-receptor agonist moxonidine.

IRAS/nischarin co-immunoprecipitates with the insulin receptor substrate 4 (IRS-4) in human embryonic kidney (HEK293) cells (Sano, Liu, Lane, Piletz, & Lienhard, 2002). It also precipitates with IRS 1, 2 and 3 in Cos7L cells, but with lower affinity (Sano et al., 2002). The binding of the imidazoline $I_1$-receptor to the IRS1-4, especially to IRS-4, opens the possibility of activation of Akt via the canonical PI3-K/Akt pathway. In fact, moxonidine activates Akt in HEK293 cells transfected with IRAS (Edwards et al., 2012). Therefore, imidazoline $I_1$-receptor agonists also signal through an imidazoline $I_1$-receptor/nischarin/IRS/PI3-K/Akt pathway (Edwards et al., 2012)(Figure 13).

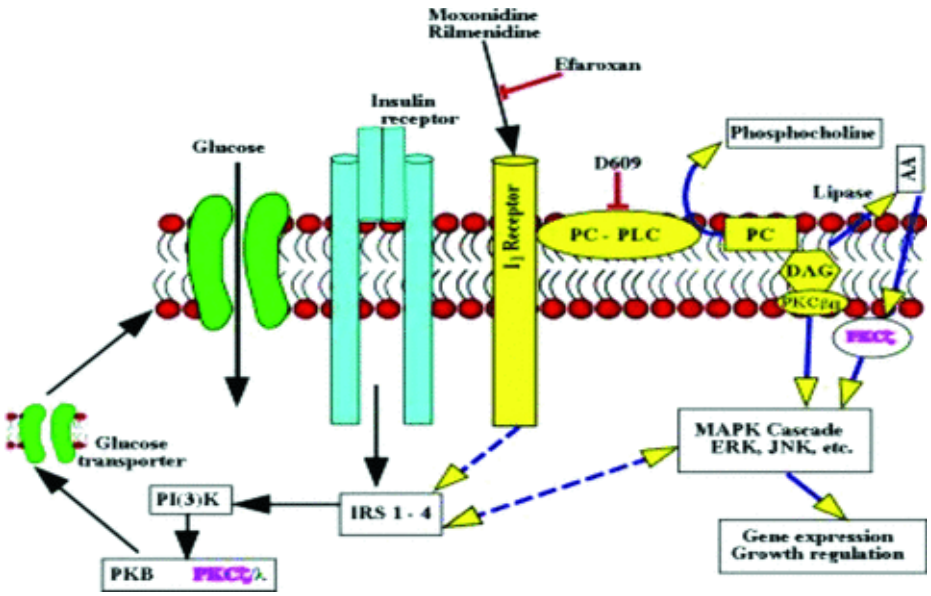


Figure 13 : Signalling through the imidazoline $I_1$-receptor. For details see text (Edwards et al., 2012). PC-PLC: Phosphatidylcholine-selective phospholipase C; AA: Arachidonic acid; DAG: diacylglycerol

Activation of the imidazoline $I_1$-receptor/nischarin/IRS/PI3-K/Akt pathway is consistent with the observation that moxonidine improves the metabolic profile of the obese SHR (SHROB), a strain with reduced insulin sensitivity (Ernsberger et al., 1999).

The current view of imidazoline $I_1$-receptor signalling is summarized in Figure 13.

In addition to the PC-PLC- and IRS-mediated pathways described so far, other cellular pathways and actions for the imidazoline $I_1$-receptor have been described in cells over-expressing nischarin. Nischarin overexpression in NIH 3T3 fibroblast cell line reduces cell migration in association with a reduction in the activation of Rac (Alahari et al., 2000), a GTP-binding protein that is part of the Rho/Rac family (Bustelo, Sauzeau, & Berenjeno, 2007). This effect has been confirmed in human breast carcinoma and colon adenocarcinoma cells, showing that migration of these cells is inhibited by nischarin through inhibition of Rac-PAK pathway (Alahari, 2003). Nischarin also interacts with LIMK1, a downstream enzyme of PAK, and this interaction inhibits LIMK1 activity and reduces cell invasion in a breast cancer cell line (Ding, Milosavljevic, & Alahari, 2008).

Overexpression of hIRAS in PC12 cells markedly reduces cell apoptosis induced by starvation, by mechanisms that include protection of mitochondrial function, and signal

through PI3-K/Akt pathway (Dontenwill, Piletz, et al., 2003). In contrast, the expression of nischarin is decreased in invasive human breast cancer and nischarin over-expression reduces proliferation of breast cancer cells in vitro and in vivo, and reduces metastasis in an animal model (Baranwal et al., 2011). These studies implicate imidazoline $I_1$-receptor/nischarin in cell growth/proliferation and show that the specific effect (induction or reduction of proliferation/cell survival) would depend on the cell type and model used.

Collectively, these studies in different cell lines reveal that imidazoline $I_1$-receptor/nischarin signalling involves enhanced MAPK phosphorylation via the PC–PLC–PKC pathway, enhances Akt phosphorylation via IRS-PI3-K pathway, and might be involved in apoptotic and anti-apoptotic processes, depending on the cell type and stimulus. In addition, imidazoline $I_1$-receptor/nischarin signals through inhibition of Rac1 and alters the distribution of α5-integrin, thus it is involved in maintenance of the ECM.

Further studies are required to investigate whether similar mechanisms mediate imidazoline $I_1$-receptor/nischarin signalling in primary cardiac cells. Furthermore, it is important to localize and identify the cellular actions of imidazoline $I_1$-receptor signalling in different cardiac cell types and in response to different growth/death inducing stimuli, such as those present in cardiac pathologies, as inhibition of fibroblast proliferation/action protects the heart from fibrosis, while inhibition of myocyte apoptosis stops/delays progression of heart disease in the hypertensive heart. Identification of these mechanisms may lead to tailoring specific cell-driven imidazoline $I_1$-receptor agonists for treatment of patients with heart disease.

# Chapter 2. Hypothesis and aims

Moxonidine, an antihypertensive sympatholytic imidazoline compound, reduces blood pressure by selective activation of non-adrenergic imidazoline $I_1$-receptors (also known as nischarin) in brainstem medulla (Edwards et al., 2012). Previous studies from our group have shown that moxonidine treatment prevents the development of LVH in SHR, associated with reduced cardiac DNA synthesis and early (after 1 week) transient increase in DNA fragmentation (Paquette et al., 2008). It is likely that the anti-proliferative and apoptotic effects have occurred in fibroblasts, as DNA fragmentation in cardiomyocytes may deteriorate cardiac function (Abbate et al., 2006). Furthermore, moxonidine exerts anti-inflammatory effects in SHR ventricles and result in normalization of left ventricular natriuretic peptide synthesis, an index of treatment efficacy (El-Ayoubi et al., 2003). Noteworthy, the effects of moxonidine have occurred in response to hypotensive as well as sub-hypotensive doses, implying a blood-pressure-independent mechanism and pointing to a local cardiac action (Paquette et al., 2008). In fact, imidazoline $I_1$-receptors have been identified in cardiac atria and ventricles (El-Ayoubi et al., 2002; El-Ayoubi et al., 2004). Cardiac imidazoline $I_1$-receptors are regulated in cardiovascular diseases and by chronic *in vivo* exposure to moxonidine (El-Ayoubi et al., 2002; El-Ayoubi et al., 2003). Also, activation of cardiac imidazoline $I_1$-receptors by moxonidine stimulates ANP release from isolated rat hearts *ex vivo* (Mukaddam-Daher et al., 2006). These studies demonstrate that cardiac imidazoline $I_1$-receptors are functional without the contribution of the central nervous system.

Therefore, based on the above studies and on i) those showing a direct anti-arrhythmic effect of moxonidine in an isolated working rabbit heart model of ischemia-reperfusion, in the absence of systemic effects, arguing in favor of local action (Wolk, Kane, Cobbe, & Hicks, 1999); ii) the role of natriuretic peptides in inhibition of myocardial cell apoptosis in hypertension and heart failure, through directly blocking apoptosis signaling pathways and/or by modulating neurohormonal factors involved in their regulation (reviewed by Mukaddam-Daher, 2006) (Mukaddam-Daher, 2006); iii) studies linking imidazoline $I_1$-receptors to the maintenance of the extracellular matrix (Amann et al., 1992) and PC12 cell survival (Dontenwill, Pascal, et al., 2003; Dupuy et al., 2004), we propose cardiac imidazoline $I_1$-receptors as a therapeutic

target. Our working hypothesis is that a**part from centrally-mediated sympatholytic function, imidazoline $I_1$-receptors in the heart may control cell growth and death. Activation of imidazoline receptors may delay the progression of cardiac pathologies into heart failure by inhibition of maladaptive proliferative signalling and downstream apoptotic pathways.**

Specifically studies have been performed to:

1. Explore *in vivo* the effects of low (sub-hypotensive) and high (hypotensive) doses of moxonidine on cardiac structure and function in two models of cardiac pathology characterized by high sympathetic output but opposite changes in cardiac structure and function, specifically in primary hypertension (SHR) and non-ischemic heart failure (cardiomyopathic hamsters).
2. Define in SHR and cardiomyopathic hamsters, the effect of *in vivo* moxonidine treatment on inflammatory markers and pathways involved in LVH and cardiac cell survival/death (MAPK and Akt).
3. Explore *in vitro* the influence of imidazoline $I_1$-receptor and its activation by moxonidine, on cell survival in inflammatory conditions. To circumvent the lack of specific imidazoline receptor agonists and antagonists, studies are performed in HEK293 cells overexpressing nischarin (murine imidazoline $I_1$-receptor).
4. Investigate *in vitro,* in the absence of central and hemodynamic effects, imidazoline $I_1$-receptor cellular localization (cardiomyocytes and fibroblasts) and regulation as well as implication in cell growth and death in response to hypertension- and heart failure-associated stimuli : norepinephrine, cytokines (IL-1$\beta$, TNF-$\alpha$), and oxidants ($H_2O_2$).

# Chapter 3. Results

## 3.1 Contributions of co-authors

The results of my work as a PhD student are presented in the following 4 papers. I have performed most of the experimental work and data analyses. I have also participated in the writing and review of all these papers. My research directors, Dr Suhayla Mukaddam-Daher (advisor) and Dr Nicolas Noiseux (co-advisor) supervised all stages of research and manuscript writing.

Other participants in the experiments and writing of the articles are:

For article 1: "**Moxonidine improves cardiac structure and performance in SHR through inhibition of cytokines, p38 MAPK, and Akt**"

Br J Pharmacol. 2011 Oct;164(3):946-57. doi: 10.1111/j.1476-5381.2011.01355.x.

Angelita Stabile: Participated in the general care and follow-up of animals used in the study and in Western blot analysis.

Georges Farah: Contributed to cardiomyocyte and fibroblast culture and Western blot analyses.

For article 2: "**Functional and molecular effects of imidazoline receptor activation in heart failure**"

Life Sci. 2011 Mar 14;88(11-12):493-503. doi: 10.1016/j.lfs.2011.01.008.

Angelita Stabile: Participated in the general care and follow-up of the animals used in the study, the biochemical measurements in tissues and in manuscript writing.

Kim Stockmeyer: Performed measurements of collagen in ventricular tissues.

Dr Abdel A. Abdel Rahman: Kindly provided nischarin antibody and performed the immunohystochemical analysis in ventricular tissues and review of the manuscript.

For article 3: "**Nischarin over-expression opposes cell-death induced by oxidative stress**"

Proceedings of the 6th European Congress of Pharmacology. 2012: 35-41. ISBN: 978-88-7587-670-8

Luis Cobos Puc: Participated in the HEK293 cell culture, Western blots and FACS.

Georges Farah: Participated in the HEK293 cell culture, preparation of cDNA, and overexpression of nischarin.

For article 4: "**Moxonidine modulates cytokine signalling and effects on cardiac cell viability**"

Eur J Pharmacol. 2014 Jul 15;740C:168-182. doi: 10.1016/j.ejphar.2014.06.047

Georges Farah: Participated in cardiomyocyte and fibroblast culture and Western blots.

## 3.2 Article 1. Moxonidine improves cardiac structure and performance in SHR through inhibition of cytokines, p38 MAPK, and Akt

Br J Pharmacol. 2011 Oct;164(3):946-57. doi: 10.1111/j.1476-5381.2011.01355.x.

# Moxonidine Improves Cardiac Structure and Performance in SHR through Inhibition of Cytokines, p38 MAPK, and Akt

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**Running title**: Improved Cardiac Structure and Performance by Moxonidine

**Abstract**

**BACKGROUND AND PURPOSE**

Regression of left ventricular hypertrophy by moxonidine, a centrally acting sympatholytic imidazoline compound, results from a sustained reduction of DNA synthesis and transient stimulation of DNA fragmentation. Because apoptosis of cardiomyocyte may lead to contractile dysfunction, we investigated in spontaneously hypertensive rats (SHR), time- and dose-dependent effects of *in vivo* moxonidine treatment on cardiac structure and function as well as on the inflammatory process and signalling proteins involved in cardiac cell survival/death.

**EXPERIMENTAL APPROACH**

12 week old SHR received moxonidine at 0, 100, and 400 $\mu g.kg^{-1}.h^{-1}$, s.c., for 1 and 4 weeks. Cardiac function was evaluated by echocardiography; plasma cytokines were measured by ELISA and hearts were collected for histological assessment of fibrosis and measurement of cardiac proteins by western blotting. Direct effects of moxonidine on cardiac cell death and underlying mechanisms were investigated *in vitro* by flow cytometry and Western blotting.

**KEY RESULTS**

After 4 weeks, the sub-hypotensive dose of moxonidine (100 µg) reduced heart rate and improved global cardiac performance, reduced collagen deposition, regressed left ventricular hypertrophy, inhibited Akt and p38 MAPK phosphorylation, and attenuated circulating and cardiac cytokines. The 400 µg dose resulted in similar effects but of a greater magnitude, associated with blood pressure reduction. *In vitro*, moxonidine inhibited norepinephrine-induced neonatal cardiomyocyte mortality but increased fibroblast mortality, through $I_1$-receptor activation and differential effects on downstream Akt and p38 MAPK.

**CONCLUSIONS AND IMPLICATIONS**

While the antihypertensive action of centrally acting imidazoline compounds is appreciated, new cardiac-selective $I_1$-receptor agonists may confer additional benefit.

**Index words**: Left ventricular hypertrophy, echocardiography, apoptosis, flow cytometry, cardiomyocytes.

**Abbreviations:** α-SMA, α smooth muscle actin; AGN192403 (2-endo-amino-3-exo-isopropylbicyclo[2.2.1]heptane); AW, anterior wall; EDD, end diastolic diameter; EDV, end diastolic volume; ESD, end systolic diameter; ESV, end systolic volume; IVRTc, isovolumic relaxation time corrected for heart rate; LVET, left ventricular ejection time; LVETc, left ventricular ejection time corrected for heart rate; LVH, left ventricular hypertrophy; LVM, left ventricular mass; MAP, mean arterial pressure; PW, posterior wall; RWT, relative wall thickness.

## Introduction

The hypertensive heart undergoes several structural and functional alterations, including increased left ventricular mass, abnormal left ventricular texture, function, and geometry, and impaired coronary reserve. Left ventricular hypertrophy (LVH), present in all patients with severe hypertension, is linked to unfavorable prognosis. A variety of deleterious consequences of cardiovascular diseases, with conditions, such as coronary heart disease, stroke, congestive heart failure and sudden death, are aggravated by LVH. It is not surprising therefore that numerous therapeutic approaches have been pursued with the aim of regressing or preventing LVH and, hence, reducing cardiovascular risk (Pierdomenico *et al*., 2008).

Many genetic, hemodynamic, neurohormonal, and growth factors, including sympathetic factors, play an important role in left ventricular remodelling (Grassi *et al*., 2009). In human hypertension, LVH correlates with increased cardiac noradrenaline spillover (Schlaich *et al*., 2003). Exposure to high concentrations of noradrenaline causes cardiomyocyte hypertrophy and loss as well as expansion of the interstitial fibroblast compartment and enhanced collagen deposition. Noradrenaline stimulates the β-adrenergic receptor pathway that includes activation of oxidative stress and the pro-inflammatory cytokines, TNF-α, IL-1β (Communal *et al*., 1998; Fu *et al*., 2004; Neri *et al*., 2007) and IL-6 (Leicht *et al*., 2003).

Moxonidine is a centrally acting antihypertensive imidazoline compound that reduces excessive sympathetic tone and peripheral vascular resistance, accompanied by reduced plasma noradrenaline concentration (Wenzel *et al*., 1998). Moxonidine treatment in patients and rats results in regression of cardiac hypertrophy (Haczynski *et al*., 2001; Paquette *et al*., 2008; Mukaddam-Daher *et al*., 2009), restoring the myocardial structure, and improving the coronary flow reserve (Mitrovic *et al*., 2001). We have reported that the anti-hypertrophic effect of moxonidine in hearts from spontaneously hypertensive rats (SHR), results from a sustained reduction of DNA synthesis and transient stimulation of DNA fragmentation and apoptotic proteins Bax and caspase3, occurring after 1 week but subsiding by 4 weeks of treatment. Cardiomyocyte apoptosis plays a causal role in the development of heart failure through progressive left ventricular wall thinning (Liao *et al*., 2004). Inhibition of cardiomyocyte apoptosis attenuates contractile dysfunction in heart failure (Foo *et al*., 2005). Accordingly, if

moxonidine-stimulated apoptosis occurred in cardiomyocytes, cardiac dysfunction would ensue. In addition, a sub-hypotensive dose of moxonidine tended to offset some of the favorable effects of the angiotensin receptor blocker, eprosartan, on cardiac function in stroke-prone SHR (Mukaddam-Daher *et al*., 2009), suggesting that higher doses may be detrimental. These studies, therefore investigated, in SHR, the time- and dose-dependent effects of chronic moxonidine treatment on cardiac structure and function as well as on the inflammatory process and signaling proteins involved in cardiac cell survival/death. In this respect, studies revealed pressure-dependent and –independent improvement of cardiac function and regression of LVH and fibrosis by moxonidine. Together with our previous finding that imidazoline $I_1$-receptors, which selectively mediate the actions of moxonidine in brainstem medulla, are also expressed in the heart (El-Ayoubi *et al*., 2002), these results led us to suggest that, in addition to central actions, the control of LVH by moxonidine may, at least in part, include direct effects on the heart. However, the participation of cardiac $I_1$-receptors in LVH regression in whole animal studies may be masked by the antihypertensive and sympatholytic effects of the drug. Therefore; the direct effect of moxonidine on cardiac cell growth and death and the implicated signalling proteins were investigated *in vitro*, on cardiomyocytes and fibroblasts in culture, in the absence of confounding mechanisms..

## Methods

For further details of the Methods please see Appendix S1.

### *Animals*

All animal care and experimental procedures for this study were approved by the Institutional Committee for Animal Protection, according to the Canadian Guidelines. SHR and normotensive Wistar-Kyoto (WKY) rats, purchased from Charles River (St. Constant, Quebec, Canada), were investigated at 12–13 weeks of age, when hypertension and LVH are established. The animals were housed at 22°C, maintained on a 12 h light-dark cycle, fed Purina Rat Chow (Ralston Purina) and had free access to tap water. They were allowed at least 3 days before experimentation.

***Experimental protocols***

Cardiac structure and function were analyzed by transthoracic echocardiography, as previously described (Mukaddam-Daher *et al.*, 2009). Then, the rats were randomly assigned to treatment with moxonidine (0, 100, or 400 $\mu g.kg^{-1}.h^{-1}$), for 1 and 4 weeks, via Alzet osmotic minipumps (model 2ML1 and 2ML4, Alzet Corporation Cupertino, CA, USA), implanted subcutaneously at the neck area, under isoflurane anesthesia, as previously described (Paquette *et al.*, 2008; Mukaddam-Daher *et al.*, 2009). The low and high concentrations of moxonidine (sub-hypotensive and antihypertensive respectively) were chosen from previous studies showing regression of LVH in hypertensive rats (Paquette *et al.*, 2008; Mukaddam-Daher *et al.*, 2009).

Rats were weighed and inspected twice per week. Food and water intake and 2 x 24 h urine output (metabolic cages) were measured after 4 weeks of treatment. Echocardiographic examination was repeated after 4 weeks of treatment. At the end of the experiment (after 1 and 4 weeks), intra-carotid blood pressure was recorded under 2% isoflurane anaesthesia. Then, blood and hearts were collected for later measurements of cytokines, molecular determinations and histopathological examination, as previously described (Paquette *et al.*, 2008; Mukaddam-Daher *et al.*, 2009).

***Histopathological examination***

Cross-sections of heart ventricles were stored in neutral buffered 4% formalin. After ethanol dehydration and embedding in paraffin, 5 μm sections were obtained and stained with hematoxylin-phloxine-saffron for cell surface measurements, or with Masson's Trichrome for measurement of collagen deposition. Microscopic visualization and photographs were obtained and measurements performed by a blinded investigator, using computer software (Micro ImageJ 1.38x, NIH, USA).

***Plasma and Cardiac Cytokines***

Plasma inflammatory cytokines (IL-1β, IL-6, and TNF-α) and anti-inflammatory cytokine (IL-10) were measured by Bio-Plex 200 System (Bio-Rad Laboratories, ON, Canada). IL-1β (Pierce/Thermo scientific, Rockford, IL) and TNF-α (Invitrogen, Camarillo, CA, USA) were also measured in left ventricles, by ELISA.

***Cardiac cell culture, flow cytometry***

Cardiomyocytes and fibroblasts were isolated from neonatal (1–2 days old) rat hearts using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp, Lakewood, NJ, USA), following the manufacturer's instructions. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, then serum deprived (DMEM + 0.1% fetal bovine serum) for 24 h to induce quiescence, before incubation with DMEM alone or with increasing concentrations of noradrenaline ($10^{-7}$ to $10^{-4}$ mol·$L^{-1}$) without and with moxonidine at $10^{-7}$ and $10^{-5}$ mol·$L^{-1}$ concentrations. Cell death was measured after 24 and 48 h, by flow cytometry after the addition of propidium iodide. In addition, cells were incubated with the above treatments for 15 min, then collected for measurement of Akt and MAPK phosphorylation, by Western blot. The participation of imidazoline $I_1$-receptors (nomenclature follows Alexander *et al.*, 2009) was identified using the $I_1$-receptor antagonist, AGN192403 ($10^{-5}$ mol·$L^{-1}$), added 15 min before addition of treatment products.

***Western blot analysis***

Cardiac proteins were measured by Western blotting using antibodies recognizing the following antigens: total and phospho-specific Akt at Ser473 (1:1000), total and phospho-specific ERK1/2 at Thr202/Tyr204 (1:1000), total and phospho-specific p38 MAPK at Thr180/Tyr182 (1:1000), total and phospho-specific c-Jun N-terminal kinase (JNK) at Thr183-Tyr185 (1:1000), (Cell signalling Technology, Inc, MA, USA), α-smooth muscle actin (α-SMA, 1:1000) and anti-GAPDH (1:10000) (Sigma-Aldrich, ON, Canada). Imidazoline $I_1$-receptor protein expression was identified in cardiomyocytes and fibroblasts using antibody raised against the murine form, nischarin (1:1000, BD Biosciences, ON, Canada). Densitometric measurements of bands was performed using ImageQuant 5.1.

***Statistical analysis***

All data obtained from SHR were compared with corresponding normotensive WKY rats and moxonidine-treated SHR. Data obtained from cultured cells were compared with corresponding DMEM-, noradrenaline- or noradrenaline + moxonidine-treated cells, as required. Comparisons were performed by one-way ANOVA (and non-parametric) test

followed by Neuman–Keuls multiple comparison test, using the computer program PRISM 4.0. Statistical significance was taken as $P < 0.05$. Data are reported as mean ± SEM.

### *Materials*

The sources of the compounds used were as follows: noradrenaline (L-(-)-norepinephrine (+)-bitartrate salt monohydrate) from Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada; AGN192403 (2-endo-amino-3-exoisopropylbicyclo[2.2.1]heptane) from Cedarlane Laboratories, Burlington, Ontario, Canada; isoflurane, from Abbott Laboratories, Saint-Laurent, Quebec, Canada.

## Results

### *Physical and hemodynamic parameters*

The effect of moxonidine on biomechanical stress in the hypertensive heart was investigated in SHR and compared with normotensive WKY rats. The SHR had higher blood pressure, LVH (left ventricular mass normalized to tibia length) and hypertrophied cardiomyocytes (Table 1), as well as higher interstitial and perivascular collagen volume (Figure 1). Moxonidine (100 mg) lowered blood pressure and heart rate after 1 week, and by 4 weeks it lowered heart rate, LVH and collagen deposition, without significant changes in blood pressure. Moxonidine at 400 mg reduced blood pressure, heart rate and LVH, at 1 and 4 weeks and collagen deposition after 4 weeks of treatment (Table 1, Figure 1). Moxonidine did not alter 24 h volume and electrolyte excretion in these rats (data not shown).

The structure and function of the left ventricle, evaluated by echocardiography (Table 2), showed that there was no significant difference in systolic functions, ejection fraction and fractional shortening among the WKY and SHR groups. Anterior wall and left ventricular posterior wall tended to increase, but relative wall thickness and left ventricular end systolic diameter were significantly higher in SHR, indicating LVH. There was no significant difference among the WKY and the three SHR groups in the ejection fraction and fractional shortening of the left ventricle, indicating maintained systolic function (Table 2). The diastolic and global functions of the heart were compromised in SHR, evidenced by delayed isovolumic relaxation time (IVRT) and higher left ventricular myocardial performance index (LVMPI) (Table 2). All systolic and diastolic function parameters were not altered within the study

duration in vehicle-treated rats. However, the two concentrations of moxonidine increased left ventricular ejection time and E wave deceleration time, and reduced E wave deceleration rate, in comparison to corresponding vehicle-treated SHR (Figure 2) or pretreatment values (not shown). In addition, 400 mg moxonidine lowered IVRT (Figure 2), and reduced relative wall thickness as well as LVMPI (Table 2), denoting decreased hypertrophy and improved global cardiac performance.

***Cytokines***

Inflammatory and anti-inflammatory cytokines were measured in plasma and hearts of SHR with and without moxonidine treatment and compared to WKY rats. Fig 3 shows that, compared to WKY, circulating TNF-α and IL-6 levels were reduced by treatment, but IL-1β levels were elevated in SHR and were further stimulated by moxonidine after 1 week, then tended to decrease after 4 weeks of treatment. Plasma IL-10 was not altered by hypertension or treatment. In contrast, left ventricular IL-1β (28 ± 1 $pg.mg^{-1}$ protein) which was not altered by hypertension, was significantly decreased (14 ± 1 and 17 ± 2 $pg.mg^{-1}$ protein; $P<0.01$) after 4 weeks of treatment with both moxonidine concentrations, respectively. Left ventricular TNF-α (25 ± 5 $pg.mg^{-1}$ protein) was not altered by hypertension or treatment (Figure S1).

***Cardiac Proteins***

To further elucidate the underlying molecular mechanism involved in signal transduction responsible for cardioprotection by moxonidine, we investigated activation of Akt and the MAPK pathway in SHR and WKY rat hearts. Figure 4 and Figure S2 show that the protein expression of P-Akt/Akt, P-ERK/ERK, and P-p38/p38 were enhanced in SHR left ventricles. Akt and p38 (Fig 4), but not ERK phosphorylation (Figure S2) were reduced by the two concentrations of moxonidine after 1 and 4 weeks of treatment. Neither hypertension nor treatment had an effect on total or phosphorylated JNK (Figure S2). Figure 4 also shows that moxonidine reduced the protein expression of α-SMA, a marker of fibroblast differentiation into contractile and hyper-secretory myofibroblasts (Petrov *et al.*, 2002).

***Cardiomyocyte and fibroblast mortality and the underlying mechanisms***

Western blot analysis revealed that neonatal rat cardiomyocytes and fibroblasts express imidazoline receptors (Figure S3). Cardiomyocytes and fibroblasts also express α- and β-

adrenoceptors whose activation by noradrenaline stimulates cell growth and death. Cardiomyocytes and fibroblasts were exposed to increasing concentrations of noradrenaline, for 24 and 48 h. Analysis by flow cytometry showed that noradrenaline at $10^{-7}$ to $10^{-4}$ mol·$L^{-1}$ increased cardiomyocyte mortality in a concentration- and time-dependent manner (data not shown) and that these effects were prevented in cells co-incubated with moxonidine at $10^{-7}$ and $10^{-5}$ mol·$L^{-1}$, indicating a protective action of moxonidine in cardiomyocytes (Figure 5). Moxonidine abolished noradrenaline-induced p38 MAPK phosphorylation, while maintaining noradrenaline-induced Akt phosphorylation (Figure 6). In contrast, incubation of fibroblasts with increasing concentrations of noradrenaline resulted in less cell death (reflecting proliferation), an effect prevented upon co-incubation with moxonidine (Figure 7). Moxonidine also abolished noradrenaline-induced p38 MAPK and Akt phosphorylation (Figure 8). The effects of moxonidine on cell death/protection and downstream signalling were opposed by the antagonist, AGN192403, demonstrating imidazoline $I_1$-receptormediated actions.

**Discussion**

Studies were performed in SHR, a genetic model of hypertension that shares many common features of human essential hypertension, including increased activity of the sympathetic nervous system and cardiac noradrenaline spillover and myocardial inflammation (Kai *et al.*, 2005; Nonaka-Sarukawa *et al.*, 2008; Kumar *et al.*, 2009). These studies reveal that 1 month treatment with moxonidine improves diastolic function and global cardiac performance in hypertensive rat hearts. The effects were associated with regression of cardiac hypertrophy and fibrosis, attenuation of cardiac IL-1β and circulating TNF-α and IL-6 as well as inhibition of Akt and p38 MAPK phosphorylation. *In vitro* studies point to a possible contribution of cardiac imidazoline $I_1$-receptors in the structural improvement, protecting against cardiomyocyte death while selectively stimulating loss of fibroblasts.

Echocardiographic/Doppler measurements revealed that systolic function was preserved in SHR but diastolic function was reduced, as shown by reduced left ventricle deceleration and ejection time and delayed relaxation time. Diastolic dysfunction, an early manifestation of cardiac dysfunction in hypertensive patients, correlates with fibrosis and

energetic functional changes in myocytes (Verma and Solomon, 2009) and independently correlates with cardiovascular risk and total mortality (Schillaci *et al.*, 2002).

Moxonidine, at both doses, did not affect systolic function but improved diastolic function parameters, even after correction to heart rate changes. The higher dose attenuated the delayed relaxation (IVRT corrected for heart rate) and reduced LVMPI, a calculated index, which includes systolic and diastolic cardiac effects independently from heart rate influence. The improved cardiac performance, which includes reduced stiffness and improved relaxation, may be due to the decrease in blood pressure and inhibition of sympathetic nerve activity and subsequent noradrenaline release by the higher dose of moxonidine (Van Kerckhoven *et al.*, 2000). However, the sub-hypotensive dose, which is lower than the concentration that did not reduce plasma noradrenaline levels in a rat model of myocardial infarction-induced heart failure (Van Kerckhoven *et al.*, 2000), also improved cardiac parameters. The effects were associated with attenuated LVH and collagen accumulation and substantial reduction in left ventricular IL-1β and circulating TNF-α and IL-6. The cytokines, IL-1β, IL-6 and TNF-α are implicated in the pathogenesis of cardiac hypertrophy and cardiomyocyte apoptosis, in vivo and *in vitro* (Bozkurt *et al.*, 1998; Dhingra *et al.*, 2007) and in stimulation of cardiac fibroblast proliferation and differentiation into activated myofibroblasts, which produce large amounts of collagens (Petrov *et al.*, 2002; Melendez *et al.*, 2010).

Hypertension-associated neurohormones, growth factors and cytokines activate MAPK signalling cascade and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. MAPKs, including ERK, p38 and JNK have distinct functions: ERK1/2 activation results in cell proliferation and survival responses, whereas JNKs and p38 are implicated in inflammation and cell growth, differentiation, migration and apoptosis (Wang *et al.*, 1998). Akt (or PKB), a serine/threonine kinase with potent anti-apoptotic action *in vitro* and in vivo, is a downstream target of PI3K and is linked to hyperplasia and hypertrophy of cardiomyocytes and increased proliferation and collagen synthesis in fibroblasts (Colombo *et al.*, 2003; Oudit *et al.*, 2004; Heineke and Molkentin, 2006). MAPK and Akt activation were evaluated in WKY and SHR hearts with and without moxonidine treatment. Our results showed no change in total or phosphorylated JNK but higher levels of phosphorylated ERK, p38 and Akt in SHR than WKY hearts. Previous studies have shown LVH and cardiac fibrosis, in association with

elevated p38 and Akt phosphorylation in several models of experimental and genetic hypertension (Behr *et al.*, 2001; Liang *et al.*, 2006; Bao *et al.*, 2007; Bartha *et al.*, 2009; Soesanto *et al.*, 2009; Esposito *et al.*, 2010). Activation of p38 in cardiomyocytes leads to a rapid onset of lethal cardiomyopathy associated with cardiomyocyte hypertrophy, interstitial fibrosis and contractile dysfunction (Liao *et al.*, 2004; Streicher *et al.*, 2010). p38 is activated by cytokines and, in turn, it is involved in the release of pro-inflammatory cytokines (Fotheringham *et al*., 2004; Li *et al.*, 2005), a mechanism by which p38 contributes to myocyte cell death (Dhingra *et al.*, 2007). On the other hand, ERK and Akt activation reduces cardiomyocyte death and protects against ischaemia-reperfusion injury (Fujio *et al.*, 2000; Liu *et al.*, 2004), but sustained activation of Akt signalling results in progression from adaptive to maladaptive hypertrophy and fibrosis (Taniyama *et al.*, 2005).

Our results showed that moxonidine treatment reduced the hypertension-associated activation of Akt and proapoptotic p38, without affecting anti-apoptotic ERK. Such changes could contribute to attenuation of hypertension-induced myocardial hypertrophy and fibrosis as well as improved cardiac performance. These results are in agreement with studies showing a protective effect of p38-specific inhibitor SB203580, regressing LVH (Behr *et al.*, 2001; Bao *et al.*, 2007) and others reporting improved cardiac performance by treatments that reduced p38 levels (Liang *et al.*, 2006; Bartha *et al.*, 2009) as well as studies showing in SHR hearts that attenuation of high Akt phosphorylation, by blocking mammalian target of rapamycin (mTOR) downstream of Akt, reduces LVH (Soesanto *et al.*, 2009).

The reduction in p38 MAPK and Akt may be indirect effects mediated by central inhibition of noradrenaline and subsequent cytokine release (Communal *et al.*, 1998; Leicht *et al.*, 2003; Fu *et al.*, 2004; Neri *et al.*, 2007), direct effects on cytokine signalling, or even downstream effects of moxonidine opposing the actions of noradrenaline, by activating cardiac imidazoline $I_1$-receptors (El-Ayoubi *et al.*, 2002). While further studies are required to evaluate these mechanisms, *in vitro* studies on cultured cardiac myocytes and fibroblasts show that moxonidine may act directly on cardiac cells, exerting opposite effects on noradrenaline-induced p38 MAPK and Akt activation in cardiomyocytes and fibroblasts, and consequently reducing cardiomyocyte mortality but stimulating fibroblast mortality. Together, these effects may, at least in part, contribute to LVH control and consequently, improved cardiac function.

In conclusion, these studies show that the benefits of moxonidine extend beyond blood pressure reduction. The previously observed early transient apoptotic effect of moxonidine (Paquette *et al.*, 2008), does not lead to deterioration of cardiac function. On the contrary, it may be therapeutic apoptosis that targets a subpopulation of susceptible cells (deBlois *et al.*, 2005) very likely macrophages and myofibroblasts; thus, reducing cytokine secretion and collagen accumulation. This anti-inflammatory effect, in addition to moxonidine-induced attenuated DNA and protein synthesis (Paquette *et al.*, 2008), may attenuate cardiac hypertrophy and fibrosis and improve cardiac function. Importantly, imidazoline $I_1$-receptors in the heart can control cardiac cell death/survival in the absence of central and haemodynamic contributions. Future new drugs or procedures, specifically targeting heart $I_1$-receptors, may prevent the development of cardiac remodelling and heart failure. While the antihypertensive action of centrally acting imidazoline compounds is appreciated, new cardiac-selective imidazoline receptor agonists with pathway-selective properties may confer additional benefit.

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**Conflict of Interest:**

None.

**References**

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edition. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Bao W, Behm DJ, Nerurkar SS, Ao Z, Bentley R, Mirabile RC *et al.* (2007). Effects of p38 MAPK Inhibitor on angiotensin II-dependent hypertension, organ damage, and superoxide anion production. J Cardiovasc Pharmacol 49: 362–368.

Bartha E, Solti I, Kereskai L, Lantos J, Plozer E, Magyar K *et al*. (2009). PARP inhibition delays transition of hypertensive cardiopathy to heart failure in spontaneously hypertensive rats. Cardiovasc Res 83: 501–510.

Behr TM, Nerurkar SS, Nelson AH, Coatney RW, Woods TN, Sulpizio A *et al*. (2001). Hypertensive end-organ damage and premature mortality are p38 mitogen-activated protein kinase-dependent in a rat model of cardiac hypertrophy and dysfunction. Circulation 104: 1292–1298.

deBlois D, Tea B-S, Beaudry D, Hamet P (2005). Regulation of therapeutic apoptosis: a potential target in controlling hypertensive organ damage. Can J Physiol Pharmacol 83: 29–41.

Bozkurt B, Kribbs SB, Clubb FJ, Jr, Michael LH, Didenko VV, Hornsby PJ *et al*. (1998). Pathophysiologically relevant concentrations of tumor necrosis factor-a promote progressive left ventricular dysfunction and remodeling in rats. Circulation 97: 1382–1391.

Colombo F, Gosselin H, El-Helou V, Calderone A (2003). Beta-adrenergic receptor-mediated DNA synthesis in neonatal rat cardiac fibroblasts proceeds via a phosphatidylinositol 3-kinase

dependent pathway refractory to the antiproliferative action of cyclic AMP. J Cell Physiol 195: 322–330.

Communal C, Singh K, Pimentel DR, Colucci WS (1998). Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation 98: 1329–1334.

Dhingra S, Sharma AK, Singla DK, Singal PK (2007). p38 and ERK1/2 MAPKs mediate the interplay of TNF-alpha and IL-10 in regulating oxidative stress and cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 293: 3524–3531.

El-Ayoubi R, Gutkowska J, Regunathan S, Mukaddam-Daher S (2002). Imidazoline receptors in the heart: characterization, distribution, and regulation. J Cardiovasc Pharmacol 39: 875–883.

Esposito G, Perrino C, Schiattarella GG, Belardo L, di Pietro E, Franzone A *et al*. (2010). Induction of mitogen-activated protein kinases is proportional to the amount of pressure overload. Hypertension 55: 137–143.

Foo RS, Mani K, Kitsis RN (2005). Death begets failure in the heart. J Clin Invest 115: 565–571.

Fotheringham JA, Mayne MB, Grant JA, Geiger JD (2004). Activation of adenosine receptors inhibits tumor necrosis factor-alpha release by decreasing TNF-alpha mRNA stability and p38 activity. Eur J Pharmacol 497: 87–95.

Fu YC, Chi CS, Yin SC, Hwang B, Chiu YT, Hsu SL (2004). Norepinephrine induces apoptosis in neonatal rat cardiomyocytes through a reactive oxygen species-TNF alpha-caspase signaling pathway. Cardiovasc Res 62: 558–567.

Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K (2000). Akt promotes survival of cardiomyocytes in vitro and protects against ischemia reperfusion injury in mouse heart. Circulation 101: 660–667.

Grassi G, Seravalle G, Quarti-Trevano F, Dell'Oro R, Arenare F, Spaziani D *et al*. (2009). Sympathetic and baroreflex cardiovascular control in hypertension-related left ventricular dysfunction. Hypertension 53: 205–209.

Haczynski J, Spring A, Przewlocka-Kosmala M, Flasinski J (2001). Effect of moxonidine on left ventricular hypertrophy in hypertensive patients. J Clin Basic Cardiol 4: 61–65.

Heineke J, Molkentin JD (2006). Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7: 589–600.

Kai H, Kuwahara F, Tokuda K, Imaizumi T (2005). Diastolic dysfunction in hypertensive hearts: roles of perivascular inflammation and reactive myocardial fibrosis. Hypertens Res 28: 483–490.

Kumar S, Seth S, Jaiswal A, Enjamoori R, Dinda AK, Ray R *et al*. (2009). Chronic beta-adrenergic activation-induced left ventricular systolic dysfunction is associated with systemic release of TNF-alpha and IL-1-beta in rats. Pharmacol Rep 61: 870–876.

Leicht M, Briest W, Zimmer HG (2003). Regulation of norepinephrine-induced proliferation in cardiac fibroblasts by interleukin-6 and p42/p44 mitogen activated protein kinase. Mol Cell Biochem 243: 65–72.

Li M, Georgakopoulos D, Lu G, Hester L, Kass DA, Hasday J *et al*. (2005). p38 MAP kinase mediates inflammatory cytokine induction in cardiomyocytes and extracellular matrix remodeling in heart. Circulation 111: 2494–2502.

Liang Q, Elson AC, Gerdes AM (2006). p38 MAP kinase activity is correlated with angiotensin II type 1 receptor blocker-induced left ventricular reverse remodeling in spontaneously hypertensive heart failure rats. J Card Fail 12: 479–486.

Liao Y, Asakura M, Takashima S, Ogai A, Asano Y, Shintani Y *et al*. (2004). Celiprolol, a vasodilatory beta-blocker, inhibits pressure overload-induced cardiac hypertrophy and prevents the transition to heart failure via nitric oxide-dependent mechanisms in mice. Circulation 110: 692–699.

Liu HR, Gao F, Tao L, Yan WL, Gao E, Christopher TA *et al*. (2004). Antiapoptotic mechanisms of benidipine in the ischemic/reperfused heart. Br J Pharmacol 142: 627–634.

Melendez GC, McLarty JL, Levick SP, Du Y, Janicki JS, Brower GL (2010). Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats. Hypertension 56: 225–231.

Mitrovic V, Hamel M, Miric M, Thormann J, Hamm C (2001). Effect of the imidazoline receptor agonist moxonidine on hemodynamics, coronary circulation, metabolic

ischemia markers and the neurohumoral system in patients with essential hypertension. Effects of moxonidine on coronary circulation. Z Kardiol 90: 953–963.

Mukaddam-Daher S, Menaouar A, Paquette P-A, Jankowski M, Gutkowska J, Gillis M-A *et al*. (2009). Hemodynamic and cardiac effects of chronic eprosartan and moxonidine therapy in Stroke Prone Spontaneous Hypertensive Rats. Hypertension 53: 775–781.

Neri M, Cerretani D, Fiaschi AI, Laghi PF, Lazzerini PE, Maffione AB *et al*. (2007). Correlation between cardiac oxidative stress and myocardial pathology due to acute and chronic norepinephrine administration in rats. J Cell Mol Med 11: 156–170.

Nonaka-Sarukawa M, Okada T, Ito T, Yamamoto K, Yoshioka T, Nomoto T *et al*. (2008). Adeno-associated virus vector-mediated systemic interleukin-10 expression ameliorates hypertensive organ damage in Dahl salt-sensitive rats. J Gene Med 10: 368–374.

Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH (2004). The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. J Mol Cell Cardiol 37: 449–471.

Paquette P-A, Duguay D, El-Ayoubi R, Menaouar A, Danalache B, Gutkowska J *et al*. (2008). Control of left ventricular mass by moxonidine involves reduced DNA synthesis and enhanced DNA fragmentation. Br J Pharmacol 153: 459–467.

Petrov VV, Fagard RH, Lijnen PJ (2002). Stimulation of collagen production by transforming growth factor-b1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 39: 258–263.

Pierdomenico SD, Lapenna D, Cuccurullo F (2008). Regression of echocardiographic left ventricular hypertrophy after 2 years of therapy reduces cardiovascular risk in patients with essential hypertension. Am J Hypertens 21: 464–470.

Schillaci G, Pasqualini L, Verdecchia P, Vaudo G, Marchesi S, Porcellati C *et al*. (2002). Prognostic significance of left ventricular diastolic dysfunction in essential hypertension. J Am Coll Cardiol 39: 2005–2011.

Schlaich MP, Kaye DM, Lambert E, Sommerville M, Socratous S, Esler MD (2003). Relation between cardiac sympathetic activity and hypertensive left ventricular hypertrophy. Circulation 108: 560–565.

Soesanto W, Lin HY, Hu E, Lefler S, Litwin SE, Sena S *et al*. (2009). Mammalian target of rapamycin is a critical regulator of cardiac hypertrophy in spontaneously hypertensive rats. Hypertension 54: 1321–1327.

Streicher JM, Ren S, Herschman H, Wang Y (2010). MAPK-activated protein kinase-2 in cardiac hypertrophy and cyclooxygenase-2 regulation in heart. Circ Res 106: 1434–1443.

Taniyama Y, Ito M, Sato K, Kuester C, Veit K, Tremp G *et al*. (2005). Akt3 overexpression in the heart results in progression from adaptive to maladaptive hypertrophy. J Mol Cell Cardiol 38: 375–385.

Van Kerckhoven R, van Veen TA, Boomsma F, Saxena PR, Schoemaker RG (2000). Chronic administration of moxonidine suppresses sympathetic activation in a rat heart failure model. Eur J Pharmacol 397: 113–120.

Verma A, Solomon SD (2009). Diastolic dysfunction as a link between hypertension and heart failure. Med Clin North Am 93: 647–664.

Wang Y, Huang S, Sah VP, Ross J, Jr, Brown JH, Han J *et al*. (1998). Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. J Biol Chem 273: 2161–2168.

Wenzel RR, Spieker L, Qui S, Shaw S, Luscher TF, Noll G (1998). I1-imidazoline agonist moxonidine decreases sympathetic nerve activity and blood pressure in hypertensives. Hypertension 32: 1022–1027.

**Figure Legends:**

**Figure 1**

Representative photomicrographs of Masson's Trichrome stained heart sections of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats with and without moxonidine treatment. (A) Interstitial, (B) perivascular collagen deposition (in blue) and (C) percentage of interstitial and perivascular collagen deposition in total area. n = 4–6 rats per group; †P < 0.01 versus WKY; ‡P < 0.05 versus vehicle; §P < 0.01 versus vehicle. Magnification 200x.

**Figure 2**

(A) Representative echo-Doppler images of diastolic function parameters in WKY and SHR. (B) Bargraphs represent data obtained after 4 weeks of study. n=10-11 rats per group. † P<0.01 versus WKY; ‡ P<0.05 , §P<0.01 vs. vehicle. IVRTc, isovolumic relaxation time corrected for heart rate; LVET, left ventricular ejection time.

**Figure 3**

Effect of moxonidine treatment on plasma cytokines. n = 9–16 rats per group. *P < 0.05, †P < 0.01 versus WKY rats; ‡P < 0.05; §P < 0.01 versus vehicle.

**Figure 4**

Western blot analysis of the effect of treatments on left ventricular Akt and p38 phosphorylation at 1 and 4 weeks of treatment. Column graphs depict the ratios of phospho-Akt to total Akt, phospho-p38 to total p38, and a-smooth muscle actin (a-SMA), compared to GAPDH (loading control) and presented as per cent of the corresponding WKY values (100%). n = 6–10 rats per group. *P < 0.05, †P < 0.01 versus WKY; §P < 0.01 versus vehicle.

**Figure 5**

(A) Representative flow cytometry and propidium iodide staining depicting total mortality of neonatal rat cardiomyocytes in culture. Cells on the right (arrow) represent the percentage of cell death out of total number of cells measured. (B) Bargraph represents cardiomyocyte mortality after 48 h incubation in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and in conditions of starvation (DMEM + 0.1% FBS) alone or

in addition to noradrenaline (NA) without and with co-incubation with moxonidine at 10-7 and 10-5 mol·L-1, without and with and $I_1$-receptor antagonist, AGN 192403 (AGN) at 10-5 mol·L-1. Data presented as per cent FBS 10%. n = 8–12 wells per treatment, from five independent cultures. †P < 0.01 versus 10% FBS; *P < 0.05 versus 0.1% FBS; §P < 0.01 versus NA; &P < 0.05 versus corresponding NA + moxonidine.

**Figure 6**

(A) Representative bands of total and phosphorylated Akt and p38 MAPK detection by Western blot in cultured neonatal rat ventricular cardiomyocytes incubated with noradrenaline (NA) alone, or upon co-incubation with moxonidine at 10-7 and 10-5 mol·L-1, without and with $I_1$-receptor antagonist, AGN 192403 (AGN) at 10-5 mol·L-1. (B) Bargraph represents ratios of phospho-Akt to total Akt and phospho-p38 to total p38, compared with GAPDH (loading control) and presented as per cent corresponding untreated cells. n = 10–12 wells per treatment from three independent cultures. †P < 0.01 versus Dulbecco's modified Eagle's medium (DMEM) + fetal bovine serum (FBS), 0.1%; ‡P < 0.05 versus NA; &P < 0.05 versus corresponding NA + moxonidine.

**Figure 7**

(A) Representative flow cytometry and propidium iodide staining depicting total mortality of neonatal rat cardiac fibroblasts in culture. Cells on the right represent the percentage of cell death out of total number of cells measured. (B) Bargraph represents fibroblast mortality after 48 h incubation in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and in conditions of starvation (DMEM + 0.1% FBS), noradrenaline (NA) alone, or upon co-incubation with moxonidine at 10-7 and 10-5 mol·L-1, without and with $I_1$-receptor antagonist, AGN 192403 (AGN) at 10-5 mol·L-1. Data presented as per cent FBS 10%. n = 8–12 wells per treatment, from five independent cultures. †P < 0.01 versus 10% FBS; *P < 0.05 versus 0.1% FBS; §P < 0.01 versus NA; &P < 0.05, +P < 0.01 versus corresponding NA + moxonidine.

**Figure 8**

(A) Representative bands of total and phosphorylated Akt and p38 MAPK detection by Western blot in cultured neonatal rat ventricular fibroblasts incubated with noradrenaline (NA)

alone, or upon co-incubation with moxonidine at 10-7 and 10-5 mol·L-1, without and with $I_1$-receptor antagonist, AGN 192403 (AGN) at 10-5 mol·L-1. (B) Bargraph represents ratios of phospho-Akt to total Akt and phospho-p38 to total p38, compared with GAPDH (loading control) and presented as per cent corresponding untreated cells. n = 10–12 wells per treatment from three independent cultures.*$P < 0.05$, †$P < 0.01$ versus Dulbecco's modified Eagle's medium (DMEM); §$P < 0.01$ versus NA; &$P < 0.05$, +$P < 0.01$ versus corresponding NA + moxonidine. FBS, fetal bovine serum.

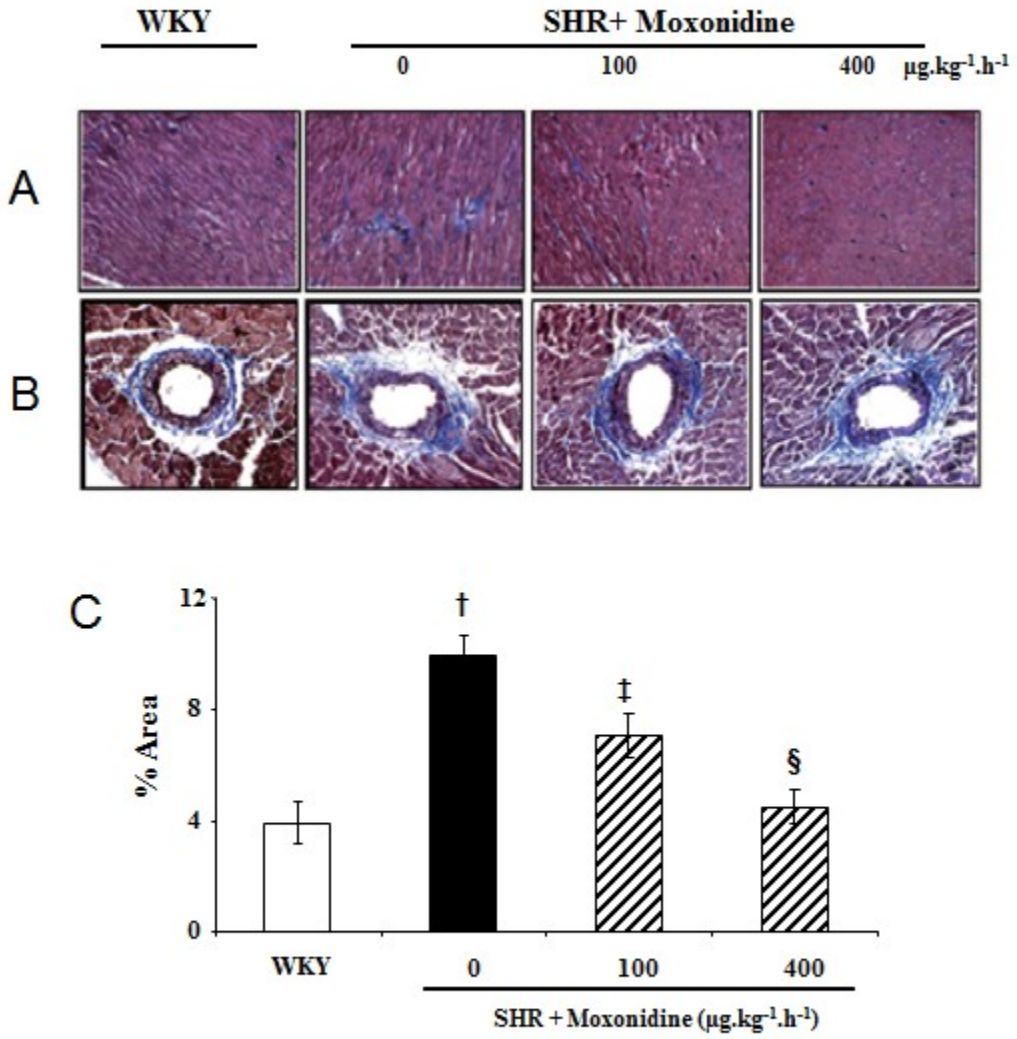
WKY
SHR+ Moxonidine
0
100
400
µg.kg-1.h-1
A
B
C
% Area
12
8
4
0
†
‡
§
WKY
0
100
400
SHR + Moxonidine (µg.kg-1.h-1)

**Figure 1**

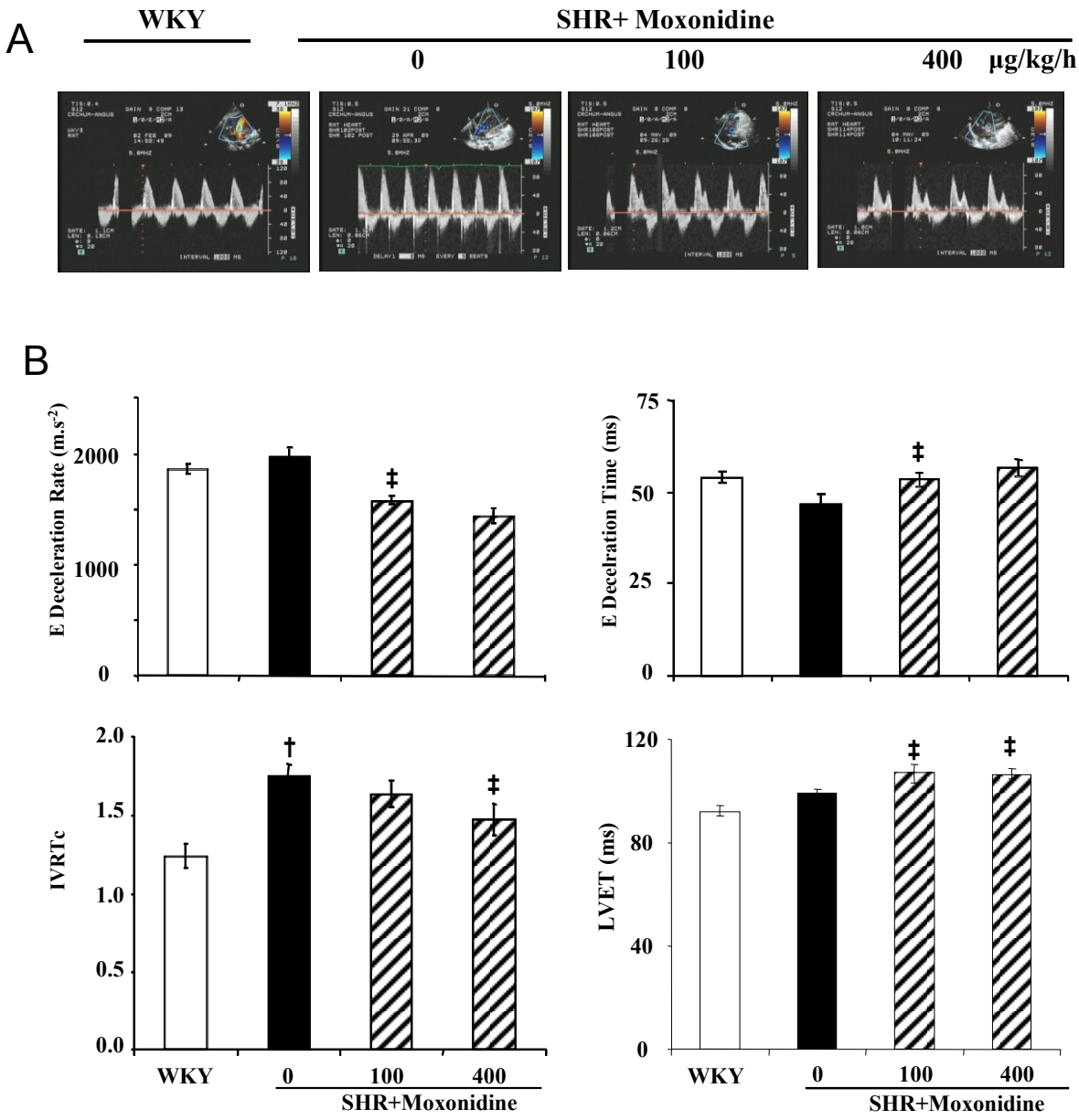
A
WKY
SHR+ Moxonidine
0
100
400 µg/kg/h
B
E Deceleration Rate (m.s-2)
2000
1000
0
‡
E Decelration Time (ms)
75
50
25
0
‡
IVRTc
2.0
1.5
1.0
0.5
0.0
†
‡
WKY
0
100
400
SHR+Moxonidine
LVET (ms)
120
80
40
0
‡
‡
WKY
0
100
400
SHR+Moxonidine

**Figure 2**

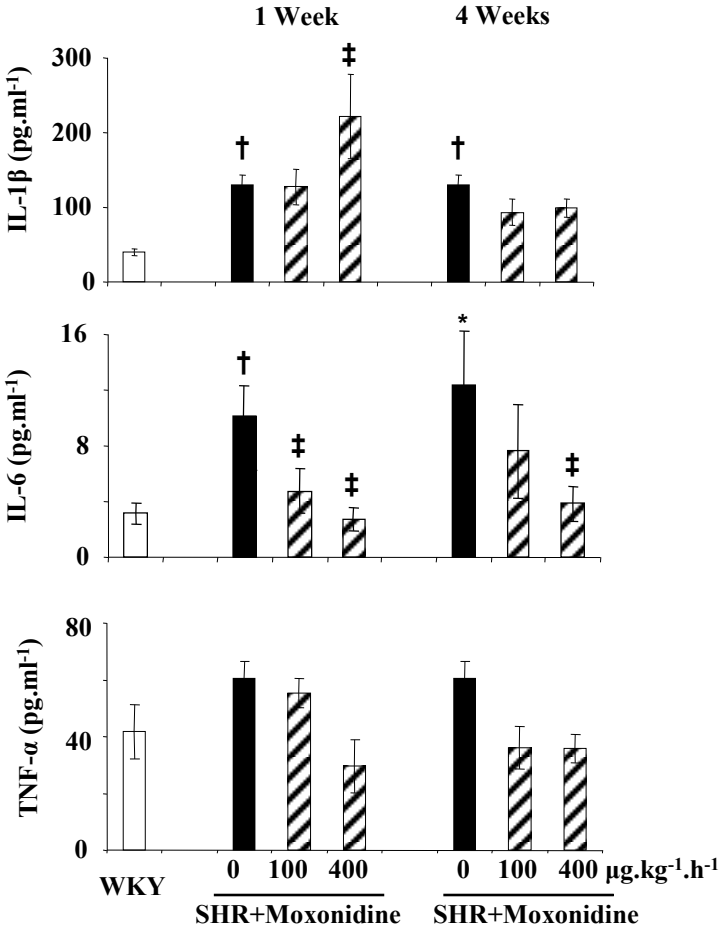
1 Week
4 Weeks
IL-1β (pg.ml-1)
300
200
100
0
†
‡
†
IL-6 (pg.ml-1)
16
8
0
†
‡
‡
*
‡
TNF-α (pg.ml-1)
80
40
0
WKY
0 100 400
0 100 400 µg.kg-1.h-1
SHR+Moxonidine
SHR+Moxonidine

**Figure 3**

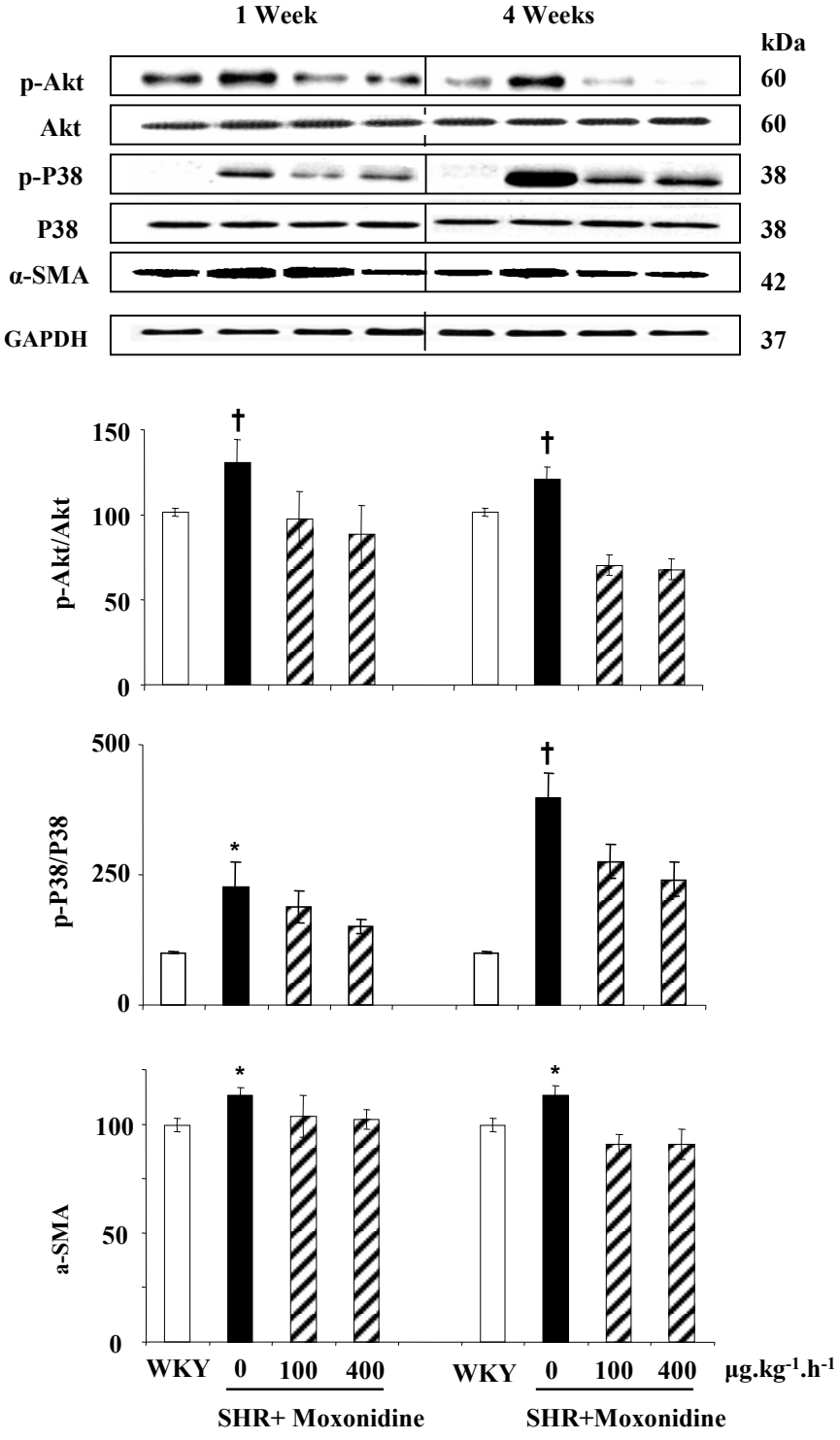
1 Week
4 Weeks
kDa
p-Akt
60
Akt
60
p-P38
38
P38
38
α-SMA
42
GAPDH
37
p-Akt/Akt
150
100
50
0
†
†
p-P38/P38
500
250
0
*
†
a-SMA
100
50
0
*
*
WKY
0
100
400
WKY
0
100
400
μg.kg-1.h-1
SHR+ Moxonidine
SHR+Moxonidine

**Figure 4**

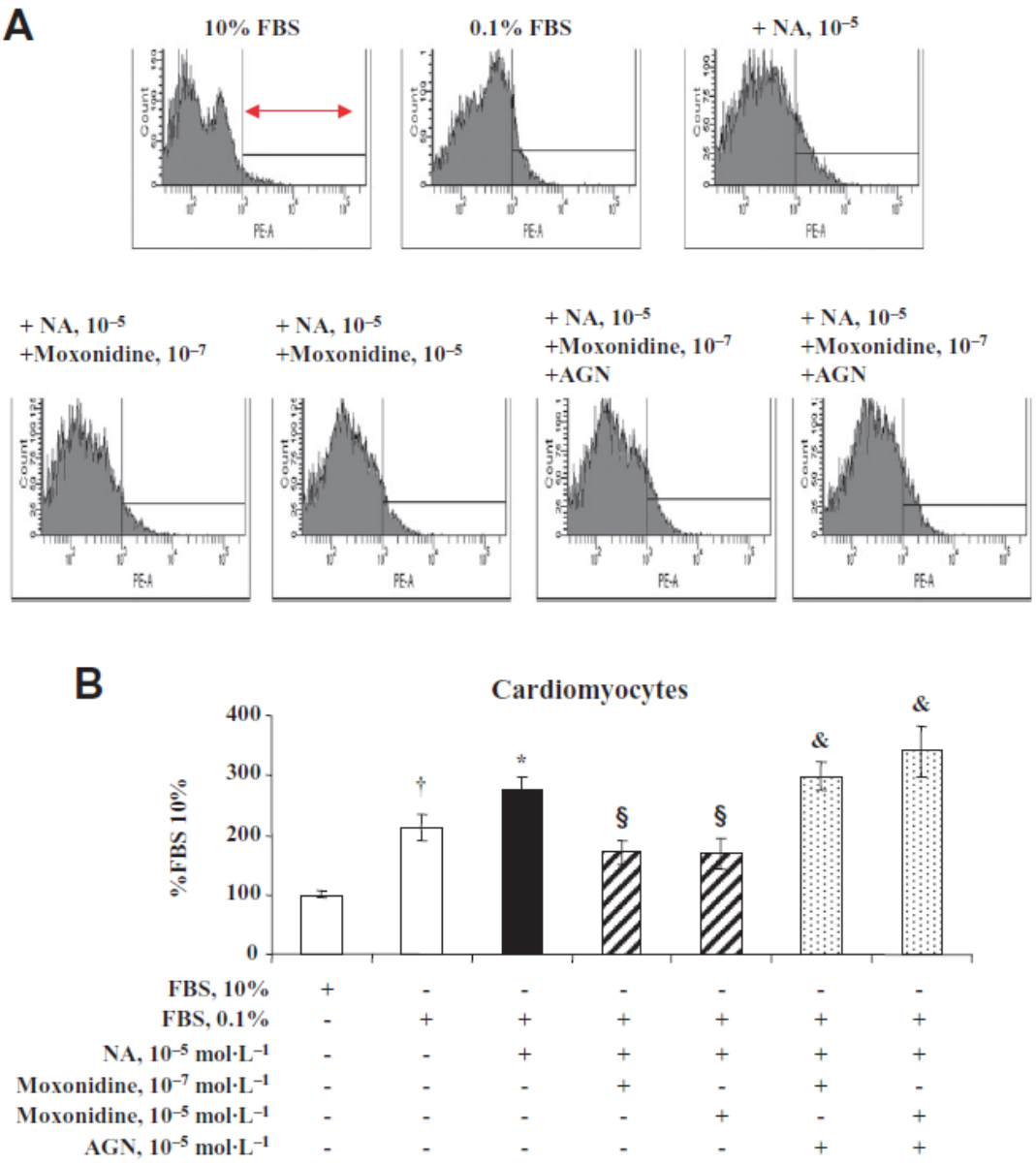
A
10% FBS
0.1% FBS
+ NA, 10–5
+ NA, 10–5
+Moxonidine, 10–7
+ NA, 10–5
+Moxonidine, 10–5
+ NA, 10–5
+Moxonidine, 10–7
+AGN
+ NA, 10–5
+Moxonidine, 10–7
+AGN
Count
PE-A
B
Cardiomyocytes
%FBS 10%
400
300
200
100
0
†
*
§
§
&
&
FBS, 10% + - - - - - -
FBS, 0.1% - + + + + + +
NA, 10–5 mol·L–1 - - + + + + +
Moxonidine, 10–7 mol·L–1 - - - + - + -
Moxonidine, 10–5 mol·L–1 - - - - + - +
AGN, 10–5 mol·L–1 - - - - - + +

**Figure 5**

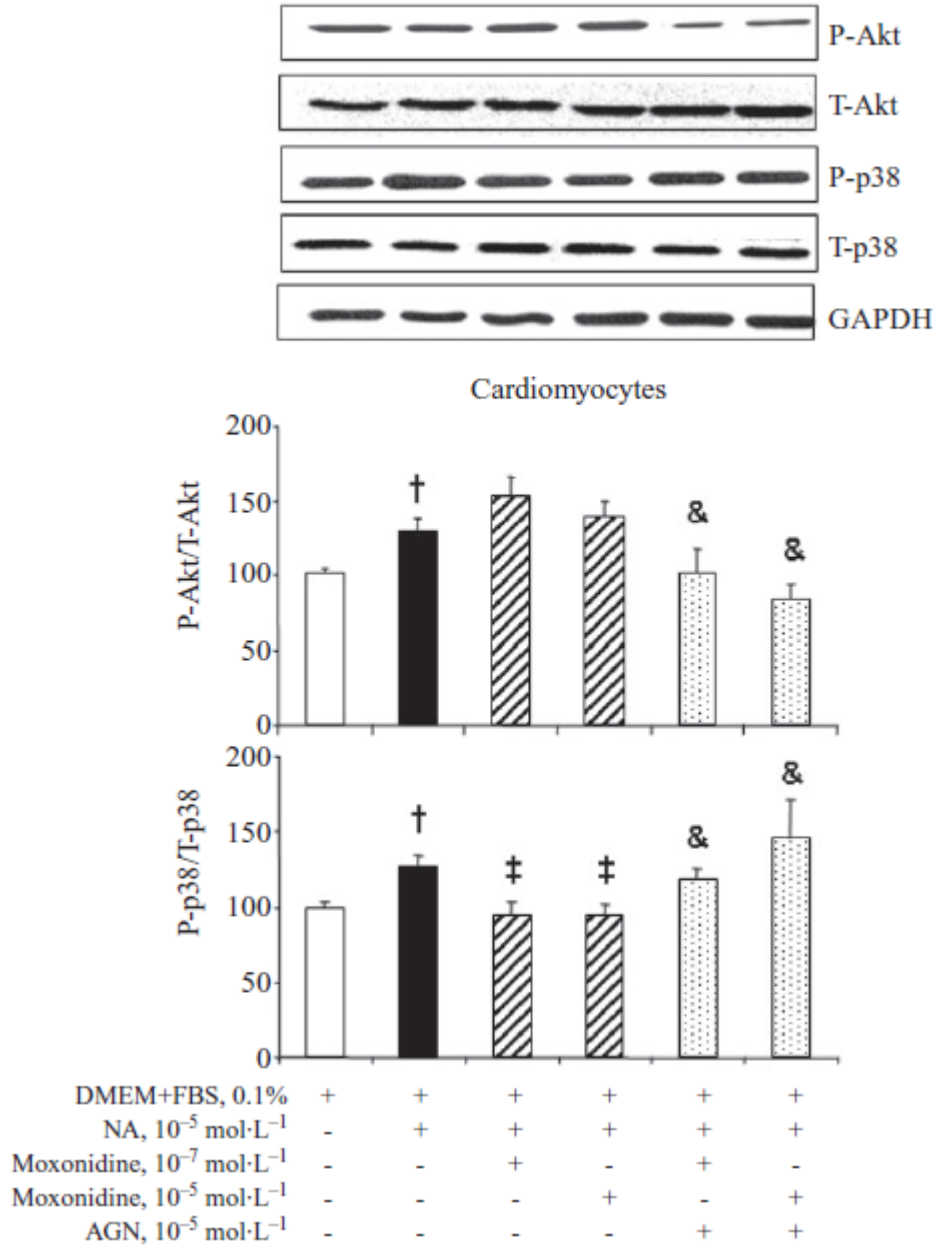
P-Akt
T-Akt
P-p38
T-p38
GAPDH
Cardiomyocytes
P-Akt/T-Akt
P-p38/T-p38
200
150
100
50
0
†
‡
&
DMEM+FBS, 0.1%
NA, 10−5 mol·L−1
Moxonidine, 10−7 mol·L−1
Moxonidine, 10−5 mol·L−1
AGN, 10−5 mol·L−1

**Figure 6**

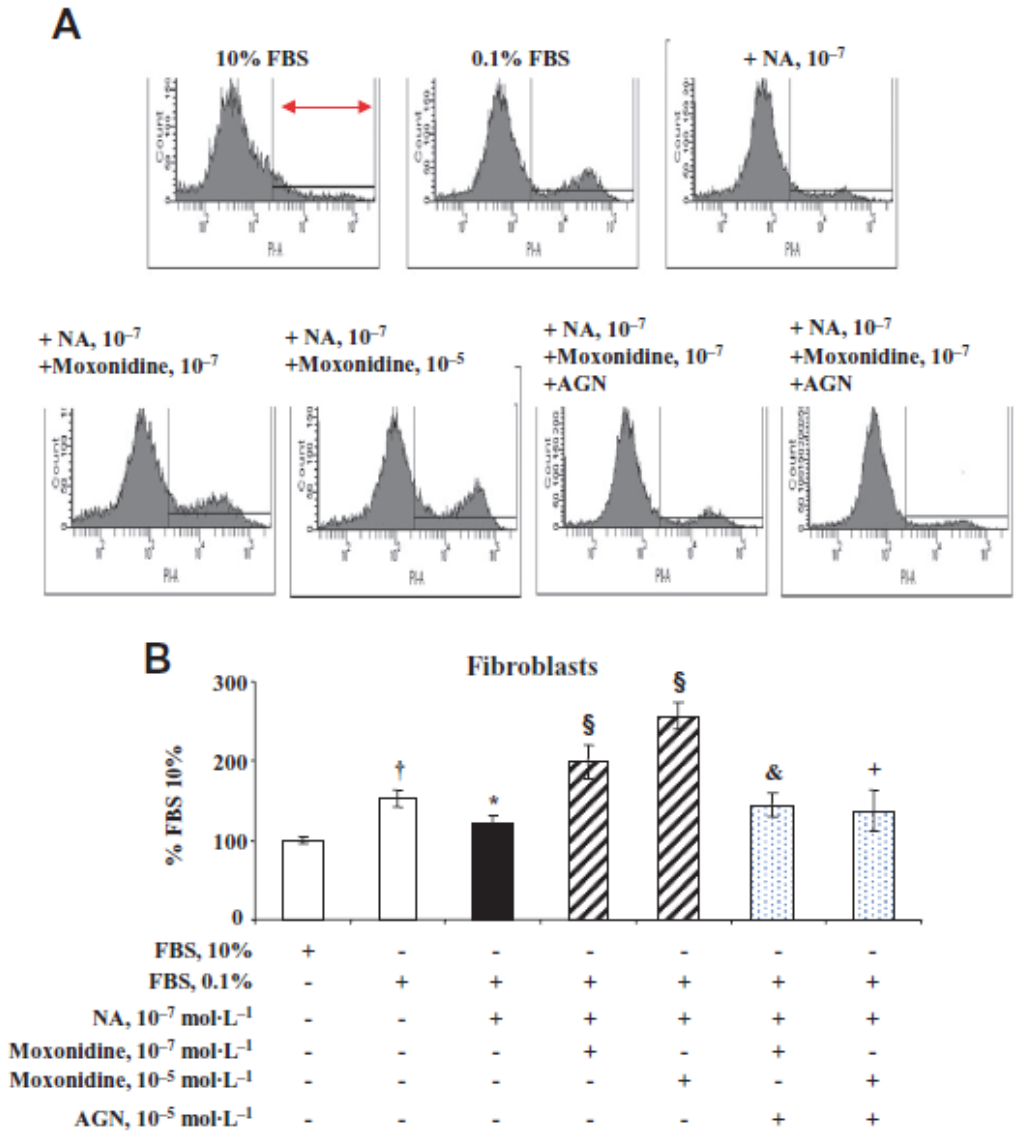
A
10% FBS
0.1% FBS
+ NA, 10–7
+ NA, 10–7
+Moxonidine, 10–7
+ NA, 10–7
+Moxonidine, 10–5
+ NA, 10–7
+Moxonidine, 10–7
+AGN
+ NA, 10–7
+Moxonidine, 10–7
+AGN
B
Fibroblasts
% FBS 10%
300
200
100
0
FBS, 10% + - - - - - -
FBS, 0.1% - + + + + + +
NA, 10–7 mol·L–1 - - + + + + +
Moxonidine, 10–7 mol·L–1 - - - + - + -
Moxonidine, 10–5 mol·L–1 - - - - + - +
AGN, 10–5 mol·L–1 - - - - - + +

**Figure 7**

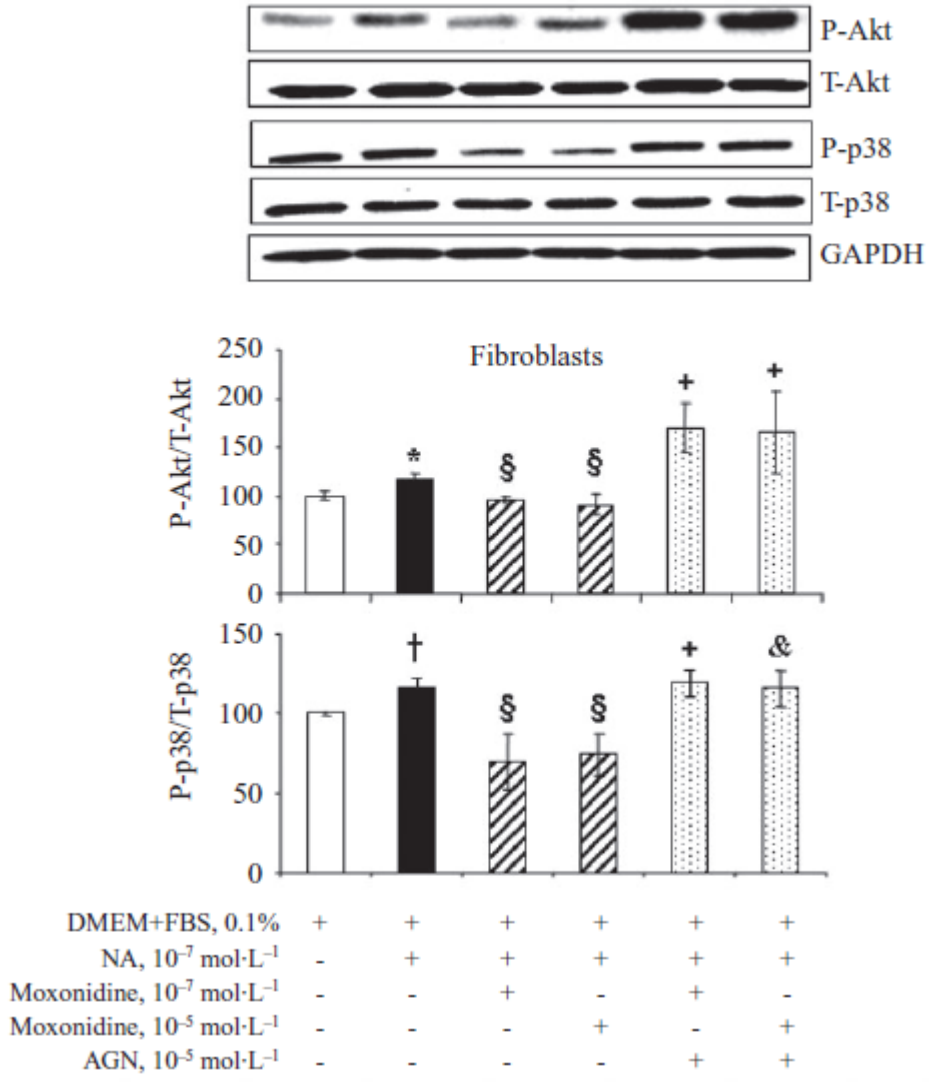
P-Akt
T-Akt
P-p38
T-p38
GAPDH
Fibroblasts
P-Akt/T-Akt
250
200
150
100
50
0
P-p38/T-p38
150
100
50
0
DMEM+FBS, 0.1%
NA, 10⁻⁷ mol·L⁻¹
Moxonidine, 10⁻⁷ mol·L⁻¹
Moxonidine, 10⁻⁵ mol·L⁻¹
AGN, 10⁻⁵ mol·L⁻¹

**Figure 8**

**Table 1: Physical and Hemodynamic parameters at 1 and 4 weeks of treatment.**

| Hemodynamic and Physical Parameters | WKY | SHR + Moxonidine (µg/kg/h) 1 Week | | | SHR + Moxonidine (µg/kg/h) 4 Weeks | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 100 | 400 | 0 | 100 | 400 |
| MAP (mm Hg) | 94±3 | 167±1* | 147±4‡ | 128±5‡ | 160±3* | 152±5 | 130±7‡ |
| Heart Rate (bpm) | 337±7 | 335±8 | 313±8 | 277±14† | 338±10 | 282±11† | 265±8‡ |
| LVM (mg) | 455±13 | 551±4* | 527±25 | 488±8‡ | 595±6* | 571±9† | 535±10‡ |
| LVH (mg/mm) | 10.1± 0.3 | 12.3±0.1* | 11.8 ±0.6 | 11.0±0.2‡ | 13.1±0.1* | 12.6±0.2† | 11.9±0.2‡ |
| CSA (µm) | 407±4 | 479±2 | 444±6‡ | 435±5† | 565±2 | 522±5‡ | 488±3‡ |

MAP: mean arterial pressure; LVM: Left ventricular mass; LVH: left ventricular hypertrophy; CSA: cross-sectional area; SHR: Spontaneously hypertensive rats; WKY: Wistar-Kyoto. *P<0.01 versus WKY; †P<0.05; ‡ P<0.01 versus corresponding vehicle.

**Table 2: Echocardiographic measurements after 4 weeks of treatment.**

| Echocardiographic Parameters | WKY | SHR + Moxonidine ($\mu g.kg^{-1}.h^{-1}$) | | |
|---|---|---|---|---|
| | 0 | 0 | 100 | 400 |
| ESD (mm) | 2.3±0.0 | 2.7±0.1 | 2.8±0.2 | 2.9±0.1 |
| EDD (mm) | 5.8±0.1 | 5.6±0.1 | 6.0±0.0 | 5.8±0.0 |
| AW (mm) | 1.7±0.1 | 1.8±0.1 | 1.7±0.0 | 1.7±0..1 |
| PW (mm) | 2.1±0.1 | 2.3±0.0 | 2.2±0.1 | 2.1±0.1 |
| RWT | 0.72±0.04 | 0.83±0.03* | 0.68±0.06† | 0.68±0.07† |
| ESV (ml) | 0.04±0.00 | 0.06±0.00* | 0.07±0.01 | 0.08±0.01 |
| EDV (ml) | 0.69±0.04 | 0.63±0.02 | 0.76±0.04‡ | 0.70±0.03 |
| Fractional Shortening (%) | 60.4±0.9 | 52.8±0.7‡ | 54.1±1.4 | 50.7±1.8 |
| Ejection Fraction (%) | 92.4±0.4 | 91.3±0.4 | 91.0±0.7 | 88.5±1.2 |
| Stroke Index ($ml.100g\text{-}^{1}$) | 0.39±0.02 | 0.29±0.01* | 0.36±0.02‡ | 0.35±0.02‡ |
| Cardiac Index ($ml.min^{-1}.100g^{-1}$) | 124±8 | 98±6* | 98±8 | 87±5 |
| LVMPI | 0.17±0.02 | 0.32±0.02* | 0.31 ± 0.02 | 0.26 ±0.02† |

*P<0.05 versus WKY; † P<0.05, ‡ P<0.01 versus corresponding vehicle.

AW, Anterior wall; EDD, end diastolic diameter; EDV, end diastolic volume; ESD, end systolic diameter; ESV, end systolic volume; LVMPI, left ventricular myocardial performance index; PW, posterior wall; RWT, relative wall thickness; SHR, spontaneously hypertensive rats; WKY, Wistar-kyoto rats.

## Supplementary data

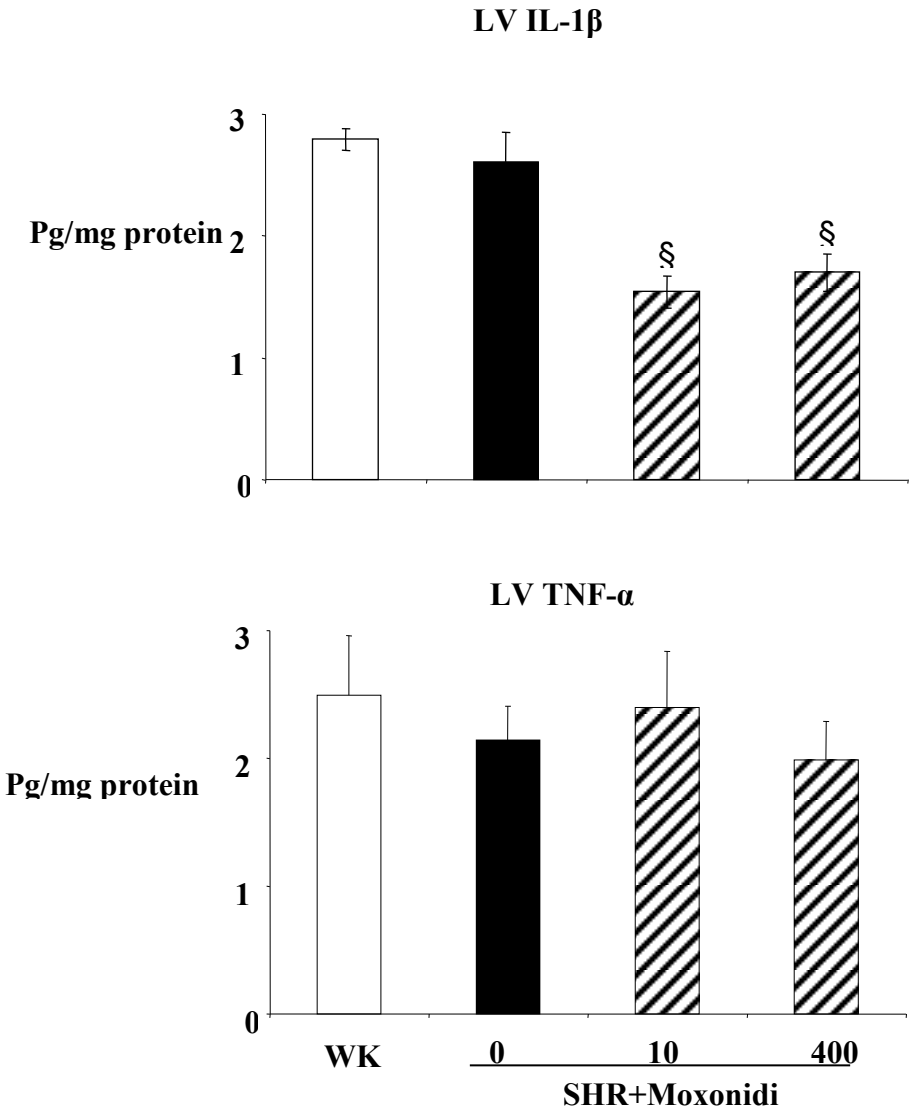


**Figure S1.** Effect of moxonidine treatment during 4 weeks on left ventricular (LV) cytokines, interleukin 1-beta and tumor necrosis factor-alpha. n=7-9 each; §P<0.01 vs. vehicle.

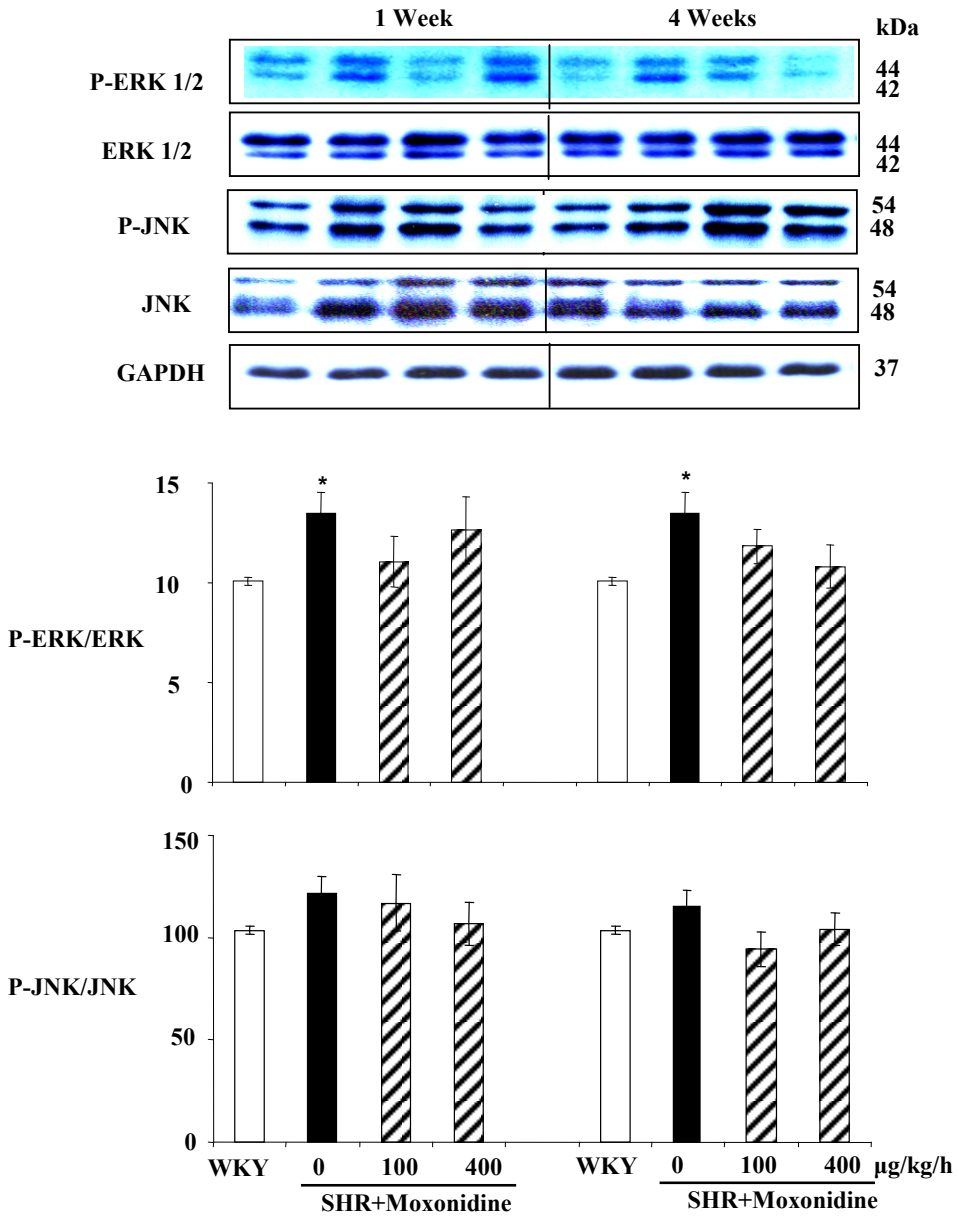


**Figure S2 :** Western blot analysis of the effect of treatments on left ventricular ERK1/2 and JNK phosphorylation at 1 and 4 week treatment. Column graph depicts the ratio of phospho-ERK to ERK, and p-JNK to JNK, normalized to GAPDH (loading control) and presented as percent WKY (100%). *P<0.05 vs. WKY. n=6-10 rats/group.

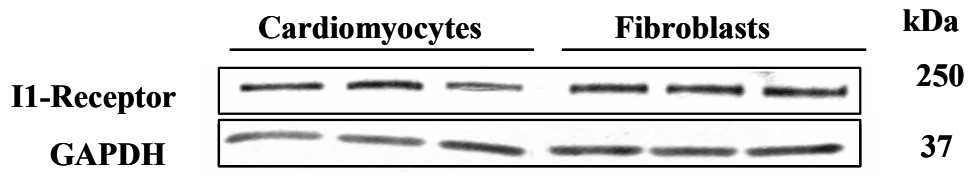


**Figure S3 :** Western blot analysis of imidazoline receptor (nischarin) in left ventricular neonatal cardiomyocytes and fibroblasts ($2^{nd}$ passage) and GAPDH (loading control). 20 µg protein were loaded.

## Detailed Methods

*Cardiac Function.* Transthoracic echocardiographic assessment of ventricular anatomy and function was performed under 2% isofluorane anesthesia, as described (Mukaddam-Daher et al. 2009; Salemi et al, 2004), using a Sonos 5500 ultrasound system (Philips, Andover, Massachusetts) with a 12 MHz transducer, placed gently on the shaved left hemithorax. The echocardiography probe was positioned to obtain short and long axis views and four and five chambers apical cardiac views.

From the cardiac short and long axis views, an M-mode tracing of the left ventricular cavity allowed measurements of interventricular septal thickness (IVS or anterior wall, AW) and posterior wall thickness as well as left ventricular end-diastolic diameter (EDD) and left ventricular end-systolic diameter (ESD). Relative wall thickness (RWT) was calculated as: (PW thickness + interventricular septal thickness)/LV interior dimension. Fractional shortening (FS) was calculated as FS = (EDD –ESD) / EDD x 100. End diastolic volume (EDV) and end systolic volume (ESV) were calculated according to Teichholz method: $[7/(2.4+ESD)] \times ESD^3$ and $[7/(2.4+EDD)] \times EDD^3$. Ejection fraction (EF) was calculated as: EF= Stroke volume/EDV. Mitral peak flow velocity in early diastole (E-wave) and during atrial contraction (A-wave) flow were recorded at the tip of mitral valve from an apical view by pulsed Doppler. Left ventricular ejection time (LVET) was measured from the beginning to the end of the aortic flow wave and the deceleration time of E wave (DT) was measured as the interval between the peak of E wave velocity and the point where the steepest deceleration slope was extrapolated to the baseline. Isovolumic relaxation time (IVRT) was recorded from the aortic outflow tract in the five chamber apical view as the interval between aortic closure and the start of mitral flow. IVRT was corrected by each R-wave and R-wave (R-R) interval time to compensate for changes in heart rate. The R-R interval was also used to calculate heart rate. Left ventricular myocardial performance index (LVMPI) is the ratio of total time spent in isovolumic activity (isovolumic contraction time and isovolumic relaxation time) to the ejection time (LVET).

All measures were acquired following the American society of Echocardiography recommendations (Cheitlin et al, 2003), by the same experienced blinded observer. Three to 5 representative cardiac cycles were analyzed and averaged for each measurement.

*Blood Pressure Measurement.* Under 2% isoflurane anesthesia, a polyethylene catheter (PE 50) filled with heparin sodium was inserted into the rat left carotid artery and pushed into the aorta to continuously monitor arterial blood pressure. The catheter was connected to a pressure transducer (Baxter Corp., ON, Canada) and signal was fed into Hewlett-Packard 783538 blood pressure monitor (Hewlett-Packard, ON, Canada). After 15 min stabilization, blood pressure values obtained over 5 minutes were averaged.

*Measurement of cytokines*

Frozen left ventricular tissue powder was homogenized in 10 mmol/L HEPES buffer (pH 7.9) containing 10 mmol/L KCl, and 0.1 mmol/L EGTA, and 0.5 mmol/L PMSF, and subsequently centrifuged at 3,000 g for 15 minutes at 4°C. Protein concentration was determined spectrophotometrically, using bovine serum albumin as a standard. Then, equal amounts of protein were used for cytokine measurements of left ventricular IL-1β and TNF-α by a quantitative sandwich enzyme immunoassay (ELISA), using microplates pre-coated with rat-specific monoclonal antibodies of either IL-1 β or TNF- α, respectively (Biosource, Montreal, QC, Canada). Results were expressed as picograms per milligram of protein. Plasma cytokines were measured with the Rat cytokines 4-Plex Kit (Bio-Rad Mississauga, ON, Canada), results were reported as pg/ml.

*Western blot analysis*

Pulverized left ventricular tissue was homogenized in NP40 buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris, pH 8.0, containing protease inhibitors)as previously described. Protein content was measured spectrophotometrically, using BSA as standard, according to the method of Bradford. Equal amounts of proteins (30 μg) separated on 10% SDS-polyacrylamide

gel were transferred to Hybond-C extra membrane (GE Healthcare Bio-Sciences, Montreal, QC, Canada). The membrane was blocked in 5% nonfat milk, 4h, at 4°C. After washing, the membrane was probed overnight at 4°C with antibodies recognizing the following antigens: rabbit polyclonal total and phospho-specific Akt-1 at Ser473 (1:1000), total and phospho-specific extracellular signal-regulated kinase (ERK1/2) (Thr202/Tyr204 1:1000), total and phosphorylated P38 mitogen-activated protein kinase (p38 MAPK) (Thr180/Tyr182 1:1000), total and phospho-specific c-Jun N-terminal kinase (JNK) (Thr183-Tyr1851:1000), (Cell signalling Technology, Inc, MA, USA), alpha smooth muscle actin (α-SMA, 1:1000). Imidazoline receptor protein expression was identified in cardiac cells, using antibody raised against the murine form, nischarin (1:1000, BD Biosciences, ON, Canada). Loading was assessed by GAPDH (anti-GAPDH, 1:10000, (Sigma-Aldrich, ON, Canada). Bound antibodies were detected by a peroxidase-conjugated anti-rabbit IgG antiserum (1:5000) or goat anti-mouse (1:5000) 1h at room temperature, and visualized by ECL-Plus chemiluminescence detection (Amersham ECL Hyperfilm). The density of the bands was quantified and compared to WKY and vehicle-treated controls.

*Histologic determination of collagen deposit*

Cross-sections of heart ventricles were stored in neutral buffered 4% formalin for 5 days. After ethanol dehydration and embedding in paraffin, 5μm slices were obtained using a microtome. Sections were stained with Masson's Trichrome. Microscopic visualization and photographs were obtained and collagen deposit measurements were performed by a blinded investigator using computer software (Micro ImageJ1.38x, NIH, USA).

*Cardiomyocyte and fibroblast Culture*

For cell culture, 18 to 21 day pregnant Sprague Dawley rats were purchased form Charles-River. The animals were maintained at the animal facility until delivery and the pups were sacrificed by decapitation at 1 to 2 days of age. The heart was excised and cardiomyocytes and fibroblasts were isolated using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp, Lakewood, NJ) following manufacturer instructions.

Cardiomyocytes were counted and plated in 12 well plates in Dulbecco's Modified Eagle Medium (DMEM) low glucose, 4mM L-Glutamine, 1 mM Sodium Pyruvate (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (HyClone Characterized Fetal Bovine Serum, Thermo Fisher Scientific Inc, Brookfield, WI) and 100 U/ml penicillin, 100 µg/ml streptomycin (P/S) (Invitrogen, Burlington, ON). At day 3, cells were starved (DMEM low glucose 0.1% FBS, P/S) for 24 h to induce quiescence, then incubated with $10^{-7}$ to $10^{-4}$ mol/L norepinephrine (L-(-)Norepinephrine (+)-bitartrate salt, Sigma-Aldrich St. Louis, MO), $10^{-7}$ to $10^{-4}$ mol/L moxonidine (Solvay Pharmaceuticals, Hannover, Germany) or NE at $10^{-7}$ and $10^{-4}$ mol/L, each in combination with $10^{-7}$ and $10^{-5}$ mol/L moxonidine, in the presence and absence of $I_1$-receptor antagonist (AGN192403).

Fibroblasts at the second passing, were plated in 12 well plates (0.5ml/well) in DMEM/F12, 2.5 mM L-Glutamine, 0.0015 mM Pyridoxine hydrochloride (Invitrogen, Burlington, ON) 10% FBS, P/S. At 70% confluence cells were starved (DMEM/F12, 0.1% FBS, P/S) for 24 h, then incubated for 48h with DMEM+0.1%FBS, norepinephrine (NE), moxonidine (M) or both (NE+M), in the presence and absence of $I_1$-receptor antagonist (AGN192403). Cardiomyocytes and fibroblasts were recovered after 24 and 48 h, for analysis by flow cytometry (FACS). In each culture, measurement of the influence of starvation alone on cell death was performed on non-starved cells, i.e. incubated in DMEM+10%FBS. Ascorbic acid (0.001%) was added to the medium to prevent norepinephrine oxidation.

Similarly treated cells were incubated for 15 min for measurement of Akt and MAPKs (total and phosphorylated) by Western blotting.

*Evaluation of cell death by Flow Cytometry (FACS):*

Cells were washed with PBS 1X, then 0.5 ml of trypsin 0.25%, 0.38 g/L, EDTA 4Na (Invitrogen, Burlington, ON) was added, and incubated for 1 min at 37ºC before cell collection and cetrifugation for 10 min. The supernatant was discarded, then, 100 µl PBS and 0.25 µg propidium iodide (Invitrogen, Burlington, ON) were added to each sample. After 15 min incubation in the dark, samples were analyzed using a BD LSRII with the software FACS DIVA version 5.0.2 (BD, Mississauga, ON).

*Biochemical determinations*

Sodium and potassium concentrations were measured in urine with a flame photometer (Instrumentation Laboratory, Lexington, MA).

**References**:

Mukaddam-Daher S, Menaouar A, Paquette P-A, Jankowski M, Gutkowska J, Gillis M-A, Shi Y-F, Calderone A, Tardif JC. Hemodynamic and cardiac effects of chronic eprosartan and moxonidine therapy in Stroke Prone Spontaneous Hypertensive Rats. *Hypertension*. 2009;53:775-781.

Cheitlin MD, Armstrong WF, Aurigemma GP, Beller GA, Bierman FZ, Davis JL, Douglas PS, Faxon DP, Gillam LD, Kimball TR, Kussmaul WG, Pearlman AS, Philbrick JT, Rakowski H, Thys DM. ACC/AHA/ASE 2003 guideline update for the clinical application of echocardiography--summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2003; 42: 954-970.

Salemi VMC, Pires M, Cestari I, Cestari IA, Picard M, Leirner A, Mady C. Echocardiographic Assessment of Global Ventricular Function Using the Myocardial Performance Index in Rats with Hypertrophy. *Artif Organs.* 2004;28: 332-337.

## 3.3 Article 2. Functional and molecular effects of imidazoline receptor activation in heart failure

Life Sci. 2011 Mar 14;88(11-12):493-503. doi: 10.1016/j.lfs.2011.01.008.

# Functional and molecular effects of imidazoline receptor activation in heart failure

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**Abstract**

**Aims:** Heart failure is a progressive deterioration in heart function associated with overactivity of the sympathetic nervous system. The benefit of inhibition of sympathetic activity by moxonidine, a centrally acting imidazoline receptor agonist, was questioned based on the outcome of a failing clinical trial. The following studies measured cardiac structure and hemodynamics and mechanisms underlying moxonidine-induced changes, in cardiomyopathic hamsters, where the stage of the disease, dose, and compliance were controlled.

**Main methods:** Male BIO 14.6 hamsters (6 and 10 months old, with moderate and advanced heart failure, respectively) received moxonidine at 2 concentrations: low (2.4 mg/kg/day) and high (9.6 mg/kg/day), or vehicle, subcutaneously, for 1 month. Cardiac function was measured by echocardiography, plasma and hearts were collected for histological determination of fibrosis and apoptosis, as well as for measurement cytokines by Elisa and cardiac proteins by Western blotting.

**Key findings:** Compared to age-matched vehicle-treated BIO 14.6, moxonidine did not reduce blood pressure but significantly reduced heart rate and improved cardiac performance. Moxonidine exerted anti-apoptotic effect with differential inflammatory/anti-inflammatory responses that culminate in attenuated cardiac apoptosis and fibrosis and altered protein expression of collagen types. Some effects were observed regardless of treatment onset, although the changes were more significant in the younger group. Interestingly, moxonidine resulted in upregulation of cardiac imidazoline receptors.

**Significance:** These studies imply that in addition to centrally mediated sympathetic inhibition, the effects of moxonidine may, at least in part, be mediated by direct actions on the heart. Further investigation of imidazolines/imidazoline receptors in cardiovascular diseases is warranted.

**Key words: Moxonidine, hamster, cardiomyopathy, apoptosis, fibrosis, echocardiography**

## Introduction

Chronic heart failure, a progressive deterioration in heart function, is characterized by an enhanced sympathetic tone, with increased circulating concentrations of catecholamines (Kaye et al. 1995) that initially tend to improve cardiac output and maintain tissue perfusion. However, long-term exposure to norepinephrine causes cardiomyocyte apoptosis, fibroblast proliferation, and cardiac fibrosis (Colucci et al. 2000). Ultimately, progressive cardiac myocyte loss, via apoptotic cell death, and the development of interstitial fibrosis contribute to progressive cardiac remodeling, arrhythmias, impaired cardiac function and development of heart failure (Foo et al. 2005; Kaye et al. 1995). Genetically engineered mice that are unable to synthesize norepinephrine exhibit less cardiac hypertrophy and preserved ventricular function after aortic banding, demonstrating the deleterious effects of sustained catecholamine excess (Esposito et al. 2002). The remodeling process is also associated with stimulated inflammatory cytokines (Fedak et al. 2005). Plasma levels of IL-1β, IL-6, and TNF-α are elevated in heart failure patients and have been linked to disease severity and poor prognosis (Aukrust et al. 1999; Rauchhaus et al. 2000). These cytokines may stimulate both anti- and pro-apoptotic pathways in the myocardium and exert negative inotropic effects, thus aggravating already impaired left ventricular function (Kubota et al. 2001; Yokoyama et al. 1993). Antagonism of excess exposure to norepinephrine within the failing heart by beta blockers (β-blocker) reduces elevated serum cytokine levels and improves cardiac function in patients with dilated cardiomyopathy (Kurum et al. 2007; Mayer et al. 2005). These findings form the cornerstone of the rationale for β-adrenoceptor antagonist (β-blocker) therapy of heart failure. However, treatment with a centrally acting sympatholytic imidazoline compound, moxonidine, did not show favorable results in a clinical trial on patients with advanced heart failure (Cohn et al 2003; Swedberg et al. 2002), despite its established benefits in hypertensive patients (Ollivier and Christen 1994) and experimental animals (Amann et al. 1992; Paquette et al. 2008). This reflects incomplete understanding of the mechanisms of action of these drugs. Better understanding of the cellular and molecular mechanisms underpinning imidazoline compounds would improve treatment of heart failure. Therefore, the aim of these studies was to evaluate cardiac structure and performance in relation to altered molecular processes induced by moxonidine, with a focus on cardiac imidazoline receptors (El-Ayoubi et al. 2002)

as well as cytokines and downstream mechanisms involved in myocardial remodeling. Because treatment benefit/adverse effects can be influenced by disease etiology, stage of heart failure, treatment dose, and/or compliance, moxonidine was given at 2 concentrations to cardiomyopathic BIO 14.6 hamsters, at 2 stages of heart failure: the hypertrophic (6 months old) and the overt heart failure (10 months old) stages.

## Materials and methods

*Experimental animals and drug treatment*

Male BIO 14.6 hamsters were purchased from Bio Breeders (Fitchburg, MA, USA) at 6 and 10 months of age (n=32, each). Age-matched F1B hamsters (n=10, each) served as normal controls. Hamsters were housed in a pathogen free environment in a temperature and light controlled room, with food and water ad libitum and were allowed 1 week to acclimatize before experimentation. Procedures were performed following the approval of the Institutional Bioethics Committee, according to the Canadian Guidelines and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Cardiac structure and function were analyzed by transthoracic echocardiography (Reffelmann and Kloner 2003). Then, cardiomyopathic hamsters were randomly assigned to treatment with moxonidine at 2 concentrations: low (2.4 mg/kg/day) and high (9.6 mg/kg/day), or normal saline vehicle, for 4 weeks, via Alzet osmotic minipumps (model 2ML4, Alzet Corporation), implanted subcutaneously under isoflurane anesthesia, as previously described (Paquette et al. 2008; Mukaddam-Daher et al. 2009). After 4 weeks of treatment, echo-Doppler measurements were repeated; then, intra-carotid blood pressure and heart rate were recorded. The hamsters were euthanized under isoflurane anesthesia. Blood was collected; hearts and lungs were excised, blotted dry, and weighed. To avoid circadian variability, all hemodynamic measurements and animal sacrifice were performed in the morning, between 8:00 and 12:00 a.m. Also, hamsters representing each group were investigated simultaneously.

The low and high concentrations of moxonidine (sub-hypotensive and antihypertensive, respectively) were chosen from previous studies showing regression of left ventricular hypertrophy in hypertensive rats (Paquette et al. 2008, Mukaddam-Daher et al. 2009).

*Cardiac function*

Echocardiography was performed under 2% isofluorane anesthesia, as previously described (Reffelmann and Kloner 2003; Mukaddam-Daher et al. 2009), using a Sonos 5500 (Philips, Andover, Massachusetts), with 12 MHz transducer, placed gently on the shaved left hemithorax. The echocardiography probe was positioned to obtain short and long axis views and four and five chambers apical cardiac views.

From the cardiac short and long axis views, an M-mode tracing of the left ventricular cavity allowed measurements of left ventricular end-diastolic diameter (EDD) and left ventricular end-systolic diameter (ESD). Percent fractional shortening (FS) was calculated as: %FS = (EDD –ESD) / EDD x 100. EDV and ESV were calculated according to Teichholz method: $[7/(2.4+ESD)] \times ESD^3$ and $[7/(2.4+EDD)] \times EDD^3$. Percent ejection fraction (EF) was calculated as: %EF= (Stroke volume/EDV) x 100. Mitral peak flow velocity in early diastole (E-wave) and during atrial contraction (A-wave) flow was recorded at the tip of mitral valve from an apical view by pulsed Doppler. The A-wave was not reported in this study because it was merged with the E wave in most of the animals. Left ventricular ejection time (LVET) was measured from the beginning to the end of the aortic flow wave and the deceleration time of E wave (DT) was measured as the interval between the peak of E wave velocity and the point where the steepest deceleration slope was extrapolated to the baseline. Isovolumetric relaxation time (IVRT) was recorded from the aortic outflow tract in the five chamber apical view as the interval between aortic closure and the start of mitral flow. IVRT was corrected by each R-wave and R-wave (R-R) interval time to compensate for changes in heart rate. The R-R interval was also used to calculate heart rate.

All measures were acquired following the American society of Echocardiography recommendations (Cheitlin et al. 2003), by the same experienced blinded observer. Three to 5 representative cardiac cycles were analyzed and averaged for each measurement.

*Blood pressure measurement*

Under 2% isoflurane anesthesia, a polyethylene catheter (PE 50) filled with heparin sodium was inserted into the hamster left carotid artery and pushed into the aorta to continuously monitor arterial blood pressure. The catheter was connected to a pressure transducer (Baxter Corp., ON, Canada) and signal was fed into Hewlett-Packard 783538 blood pressure monitor (Hewlett-Packard, ON, Canada). After 15 min stabilization, blood pressure values obtained over 5 minutes were averaged.

*Histopathologic measurements*

Hearts were prepared for histologic determinations as previously described (Paquette et al. 2008, Mukaddam-Daher et al. 2009). Sections were stained with hematoxylin, phloxine, saffron (HPS) for microscopic cell surface measurements, using computer software (Micro Dimension Version 1.01, 1993). Cell surface was measured in 10 different areas of the sections. Only cardiac myocytes with nuclei centrally located were included in the analysis. Other heart sections were stained with Masson's Trichrome for collagen measurement. The degree of fibrosis was calculated as percentage of the fibrotic area (blue) in relation to the total heart area by computer-aided image analysis of tissue sections (Mukaddam-Daher et al. 2009). At least 10 fields at 200-fold magnification were captured and assessed in all the samples. To detect apoptotic cells, heart sections were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) using ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (CHEMICON International, Cedarlane Laboratories, ON, Canada). Cardiac imidazoline $I_1$-receptors were detected in heart sections using anti-nischarin (murine imidazoline $I_1$-receptor) antibody (1:5000). NIH Image J 1.43 was used to calculate the integrated density, which takes into account the number of receptors as well as the intensity of the signal (Zhang and Abdel-Rahman 2008).

*Cytokine measurement in plasma and hearts*

Plasma inflammatory cytokines (IL-1β, IL-6, and TNF-α) and anti-inflammatory cytokines (IL-10) were measured by Bio-Plex 200 System (Bio-Rad Laboratories, ON, Canada). IL-1β (Pierce/Thermo scientific, Rockford, IL) and TNF-α (Invitrogen, Camarillo,

CA) were also measured in left ventricles, by enzyme-linked immunosorbent assay (ELISA) kits following the recommendations of the manufacturers.

*Western Blotting*

Left ventricles were homogenized in NP-40 buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris Buffer, pH 8.0) supplemented with protease and phosphatase inhibitors, as previously described (Paquette et al. 2008, Mukaddam-Daher et al. 2009). The samples were then centrifuged and protein content in the supernatant was determined using the Bradford assay.

To avoid inter-assay variation, equal amount of protein from samples representing all groups were simultaneously subjected to SDS-PAGE on a 10-12.5% polyacrylamide gel, and proteins were electroblotted on a Pure Nitrocellulose Membrane (Bio-Rad Laboratories, ON, Canada). After blocking, the membranes were incubated with antibodies against Bax (1:1000, Cell Signaling, Pickering, Ontario, Canada) or Bcl-2 (1:1000, BD Transduction, ON, Canada), collagen I and III (1:1000), and alpha smooth muscle actin (α-SMA, 1:5000), with GAPDH (1:10,000), as loading control, followed by horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences, Quebec, Canada). Signals were visualized by ECL Plus Western blotting Detection System (Amersham).

*Statistical analysis*

Results are expressed as means ± SEM. Comparisons between groups were performed by one way ANOVA followed by Neuman-Keuls Multiple Comparison Test, using the computer program PRISM 4.0. Values with $p<0.05$ were considered statistically significant.

**Results**

*Hemodynamic and physical parameters*

The hemodynamic and physical parameters of 6- and 10-month-old normal and cardiomyopathic (BIO 14.6) hamsters are presented in Tables 1 and 2, respectively. Compared to corresponding age-matched normal controls, BIO 14.6 hamsters had lower body weight and higher left ventricular hypertrophy (LVH), presented as left ventricular weight normalized to

body weight or to tibia length. BIO 14.6 hamsters also showed significantly lower mean blood pressure, but similar heart rate (Tables 3 and 4). In addition to LVH, 10-month-old BIO 14.6 hamsters had hypertrophied right ventricles and right and left atria. All these parameters remained unaffected by moxonidine treatment, in both age groups (Tables 1 and 2), except heart rate, which was significantly decreased by the high moxonidine (9.6 mg/kg/day) dose (Tables 3 and 4).

*Cardiac performance*

To evaluate the impact of moxonidine on cardiac performance, echocardiography was performed on all hamsters before and after 1 month of treatment. Fig. 1 shows representative M-mode recordings of diastolic and systolic left ventricular (LV) diameters. Measurements taken from these views revealed age-dependent deterioration in systolic function. The BIO 14.6 hamsters showed age-dependent cardiac chambers dilatation, wall thinning, and reduced myocardial function represented by fractional shortening (%FS) and ejection fraction (%EF) (Fig. 1). The LV fractional shortening and ejection fraction of untreated BIO 14.6 hamsters further decreased over 4 weeks, thereby indicating that contractile dysfunction was progressing. This deterioration was prevented by the high dose of moxonidine in 6-month-old hamsters and by both, low and high doses, in 10-month-old hamsters (Fig. 1). End diastolic volume (EDV) and end systolic volume (ESV) decreased by the high dose only in young hamsters (Tables 3 and 4).

Doppler measurements (Tables 3 and 4) revealed that the low dose moxonidine had no effect on diastolic function, but the higher dose prevented the progressive changes in ventricular deceleration time (DT), deceleration rate, and ejection time (LVET). Isovolumetric relaxation time corrected for heart rate (IVRTc) was not significantly altered by heart failure or treatment. Noteworthy, the improvement in diastolic function remained significant after correction for heart rate changes, but only in the young group.

*Myocardial fibrosis and myocyte apoptosis*

Histological analysis revealed that ventricular cardiomyocyte cross-sectional area was bigger in cardiomyopathic hamsters than corresponding normal controls, and was reduced by moxonidine, in both age groups (Tables 1 and 2). TUNEL analysis (Fig. 2) revealed very low

level of apoptotic cardiomyocytes in normal hamster hearts, but apoptotic cells were 7.4±0.5% and 8.3±0.2% of total cardiomyocyte number measured in young and old cardiomyopathic hamsters, respectively. Apoptotic cell number was significantly attenuated by high dose moxonidine, in both age groups (Fig. 2). Moxonidine did not alter the high expression of the pro-apoptotic protein Bax, but increased the expression of the anti-apoptotic protein, Bcl-2, in both groups. These changes resulted in significantly higher Bax to Bcl-2 ratio in cardiomyopathic hamsters and in significant lowering by treatment, consistent with anti-apoptotic action (Fig. 3).

The percentage of collagen deposition in 6- and 10-month-old BIO 14.6 hamsters was 2 and 3 fold higher than that in normal controls. Treatment with high dose moxonidine attenuated myocardial collagen deposition only in the older group (Fig. 4). Western blot analysis of collagen forms showed that both collagen I, and more so, collagen III were higher in heart failure hamsters than normal controls (Fig. 5). High dose moxonidine reduced collagen III in the young hamsters, resulting in higher collagen I to collagen III ratio, but treatment did not alter this balance in the old hamsters. Moxonidine reduced elevated α-SMA, a marker of fibroblast differentiation into myofibroblasts, in the young, but not the old group (Fig. 6).

*Plasma and LV cytokines and cardiac proteins*

Fig. 7 shows that serum pro-inflammatory cytokine IL-1β increased upon treatment, in both age groups, with highest levels detected in high dose moxonidine-treated BIO 14.6 hamsters. In contrast, left ventricular IL-1β was inhibited by high dose moxonidine in both age groups. Low levels of serum TNF-α were detected in normal and cardiomyopathic hamsters but were below detectable levels in moxonidine-treated BIO 14.6 hamsters. Left ventricular TNF-α was not affected by low, but significantly inhibited by high moxonidine, in both groups. Serum IL-6 was elevated in BIO 14.6 and significantly reduced by treatment, in both groups. On the other hand, anti-inflammatory IL-10 levels, which were lower in BIO 14.6 than normal 10-month old hamsters, tended to increase by high moxonidine, but values did not reach statistical significance. Downstream, p65 NF-κB activation (p-NF-κB to NF-κB ratio) and iNOS, high in cardiomyopathic hamster hearts, were reduced by treatment (Fig. 8). These changes were associated with upregulation of imidazoline $I_1$-receptors in the heart and the up-

regulation was dose-dependent in the 6-month-old hamsters (Fig. 9), suggesting increased responsiveness to treatment.

**Discussion**

These studies are the first to dissect the influence of chronic moxonidine/imidazoline receptor activation on cardiac proteins and cytokines in heart failure. The results provide evidence that moxonidine improves cardiac performance and exerts anti-apoptotic effect with differential inflammatory/anti-inflammatory responses that culminate in reduced cardiac apoptosis and fibrosis and altered protein expression of collagen types. Some effects are observed regardless of treatment onset, although the changes are more significant in the younger group. Interestingly, moxonidine results in upregulation of cardiac imidazoline receptors, implying that in addition to centrally mediated sympathetic inhibition, the effects may, at least in part, be mediated by direct actions on the heart.

The BIO14.6 cardiomyopathic hamster with a delta-sarcoglycan gene mutation is a representative model of hypertrophic dilated cardiomyopathy well characterized and extensively used experimentally to study heart failure. This strain presents a defined progression to heart failure with age, with hypertrophy starting around 4-5 months, cardiac decompensation beginning around 6 months, and as early as 10 months, the hamsters develop heart failure (Bajusz 1969; Kawada et al. 1999). These hamsters also demonstrate characteristic autonomic imbalance, as observed in human cardiomyopathy (Giudice et al. 2000; Sakamoto et al. 1997). Thus, the BIO14.6 cardiomyopathic hamster offers a convenient and reproducible model that allows evaluation of cardiac function and the effect of treatment, at the onset of LV dysfunction as well as in overt heart failure, especially when studies involve the autonomic nervous system.

Moxonidine is an antihypertensive imidazoline compound, highly effective in reversing cardiac hypertrophy and protecting myocardial structure in hypertensive patients and rats (Amann et al. 1992; Paquette et al. 2008). Moxonidine reduces sympathetic outflow and circulating levels of catecholamines by selective binding to imidazoline $I_1$-receptors, primarily

located in the brainstem medulla (Haxhiu et al. 1994; Mobini et al. 2006; Motz et al. 1998; Van Kerckhoven et al. 2000).

The BIO 14.6 hamsters presented low blood pressure and age-dependent impaired systolic and diastolic functions, evidenced by reduced fractional shortening and ejection fraction, as well as reduced E wave deceleration time and faster deceleration rate. One month treatment with moxonidine did not significantly affect blood pressure. These findings fully agree with the reported inability of moxonidine and other centrally acting drugs to produce hypotension, particularly in presence of low or normal blood pressure (Motz et al. 1998; Mukaddam-Daher and Gutkowska 2000). It is imperative to note that, even in the absence of hypotension, centrally acting drugs cause significant suppression in cardiac sympathetic activity (Mobini et al. 2006; Van Kerckhoven et al. 2000). This latter effect might contribute, at least partly, to the favorable cardiac effects by reducing heart rate and improvement of myocardial compliance. Lower heart rate in moxonidine-treated animals in the present study would be beneficial to the failing heart, because it may lead to longer diastolic filling phase. A 10% decrease in heart rate was associated with a 20% increase in stroke volume in heart failure patients treated with oral moxonidine for 3 months (Motz et al. 1998). Importantly, after correction for heart rate in the present study, the deceleration rate of E wave tended to be maintained in the old hamsters, and was significantly improved in the young hamsters. This finding reflects improved ventricular compliance, independently from heart rate, most likely due to the observed structural modification of the heart (Stilli et al. 2006).

The high dose of moxonidine prevented the deterioration in ejection fraction observed in untreated young hamsters; and both, low and high doses prevented the deterioration in ejection fraction in old hamsters. Although the magnitude of these changes appears small, representing 13% and 8% in young and old hamsters, respectively; it may be physiologically significant. Treatment that resulted in an absolute increase of 5% in EF was associated with improved walking distance and respiratory stability and reduced nocturnal respiratory events, in patients with advanced heart failure (Oldenburg et al. 2010). Even a smaller increase in EF was associated with significantly improved exercise capacity and VO2max in patients with dilated cardiomyopathy (Doesch et al. 2010).

Moxonidine treatment did not affect cardiac hypertrophy in the BIO 14.6 hamsters. However, it reduced cardiomyocyte size, resulted in lower number of TUNEL-detected apoptotic cardiomyocytes and reduced the significantly elevated Bax to Bcl-2 ratio. An increased expression of pro-apoptotic Bax to anti-apoptotic Bcl-2, an index of apoptosis, has been reported in several animal models of heart failure (Dent et al. 2007; Leri et al. 1998; Moorjani et al. 2007). In the present study, the expression of Bax was not significantly affected by moxonidine treatment in both age groups. However, the expression of Bcl-2 was increased by moxonidine; and interestingly, the effect was more evident in response to low dose moxonidine in the younger group. These findings demonstrate enhanced beneficial effects on left ventricular cell apoptosis when moxonidine is given earlier in heart failure development. There is increasing evidence that interrupting or reducing apoptosis can attenuate or prevent heart failure, regardless of the mechanisms involved (Bristow 1998; Foo et al. 2005; Kawai et al. 2004).

Moxonidine also attenuated collagen deposition and altered collagen types. Although the proportion of type I and III collagens determines stiffening/resistance and elasticity, respectively, collagen I to collagen III ratio was higher in the young hamster hearts after moxonidine treatment. There are conflicting reports about the significance of altered collagen types in heart failure (Pauschinger et al. 1999; Timonen et al. 2008). But the fact that the young hamsters had improved pumping capacity after treatment leads to the suggestion that preferential activation of collagen I and inhibition of collagen III is beneficial, most likely by decreasing or preventing further dilatation of the dilated failing heart.

The anti-apoptotic and anti-fibrotic effects of moxonidine may be due to its effect on cytokines. Circulating proinflammatory cytokine (IL-1β, IL-6 and TNF-α) levels increase with progression of heart failure (Aukrust et al. 1999; Timonen et al. 2008). Cytokines and other cytokine-related inflammatory factors contribute to cardiac remodelling by initiating or stimulating the cytokine cascade, inducing apoptosis/anti-apoptosis, as well as by regulating collagen turnover (Siwik et al. 2000). Cytokines induce the differentiation of fibroblasts into myofibroblasts, which have a higher activity for collagen production than fibroblasts (Petrov et al. 2002). In the present study, untreated heart failure hamsters had elevated IL-6 and TNF-α and more collagen deposition and protein expression of α-SMA, indicating enhanced

differentiation of cardiac fibroblasts into myofibroblasts, which secrete collagens. These parameters were reduced by moxonidine, which also attenuated left ventricular TNF-α, IL-1β, NF-κB p65 activation, and iNOS expression, demonstrating anti-inflammatory, anti-oxidative, and antifibrotic actions. On the other hand, moxonidine increased IL-1β circulating levels. The functional consequences of elevated IL-1β on the failing hamster heart warrant further investigation; however it was recently shown that a sub-inflammatory low level of IL-1β maintains adaptable compensation hypertrophy and inhibits interstitial fibrosis (Honsho et al. 2009).

The beneficial effects of moxonidine may be secondary to inhibition of SNS activity. The maintenance of ejection fraction after chronic moxonidine treatment may be consequent to decreased left ventricular end diastolic volume, and which was shown in patients to correlate with the decrease in plasma norepinephrine (Cohn et al. 2003). In addition, antagonism of excess exposure to norepinephrine within the failing heart by beta blockers reduces elevated serum cytokine levels and improves cardiac function in patients with dilated cardiomyopathy (Kurum et al. 2007; Mayer et al. 2005). The mechanisms may include direct actions on adrenergic receptors in immune cells and T and B lymphocytes, the primary source of cytokines (reviewed by Nance and Sanders 2007) as well as on adrenergic receptors in cardiomyocytes and fibroblasts, which can produce cytokines. However, most of the effects were also observed when moxonidine was given at a non-sympatholytic concentration (Van Kerckhoven et al. 2000), implying contribution of mechanisms other than centrally-mediated sympatholysis. In this regard, moxonidine may have exerted direct actions on up-regulated cardiac imidazoline $I_1$-receptors and may have contributed to the anti-apoptotic and anti-inflammatory effects as well as the inotropic effect, essential to support the failing heart. It remains to be mentioned that $I_1$-receptor up-regulation is unlikely influenced by inhibition of SNS activity, because a similar treatment in SHR normalized up-regulated receptors (El-Ayoubi et al. 2003). The mechanisms that lead to imidazoline $I_1$-receptor up-regulation and the influence and long term consequences of imidazoline $I_1$-receptor regulation, especially on myocardial metabolism (Mobini et al. 2006) and natriuretic peptides (El-Ayoubi et al. 2003) remain to be investigated.

Some limitations of the study have to be acknowledged. In the present study, norepinephrine levels were not measured. However, moxonidine was administered to heart failure hamsters at a low concentration (2.4 mg/kg/day) which is within the range that did not reduce plasma norepinephrine levels, and a high concentration (9.6 mg/kg/day), which is much higher than the 6 mg/kg/day that significantly reduced plasma norepinephrine levels in a rat model of MI-induced heart failure (Van Kerckhoven et al. 2000). Accordingly, at least in the high dose-treated group, plasma norepinephrine levels are expected to be reduced. Also, although low blood pressure in cardiomyopathic hamsters may be secondary to heart failure-reduced cardiac output, isoflurane anesthesia may have also contributed to it. Taking that in consideration, both, cardiomyopathic and normal hamsters were investigated similarly and in parallel. Finally, because several cardiac function parameters are load-dependent, acquiring invasive pressure-volume loop measurements would be helpful. Importantly, improved cardiac performance, including reduced heart rate, reveal treatment efficacy. A study with longer treatment duration and measurement of animal survival would have offered important findings, although 1 month treatment is relatively long compared to the hamster lifespan.

## Conclusion

We obtained evidence that moxonidine is effective, preserving and/or improving cardiac performance, by slowing/preventing the degenerative disease process in severe but, more so, in compensatory heart failure. However, despite the favorable outcome of moxonidine when given in early stages of heart failure to a homogenous and well controlled animal model, extrapolation to patients should be taken with caution. Furthermore, these studies do not aim at improving the chances for moxonidine in heart failure treatment, but at gaining insight on the cellular and molecular mechanisms of imidazoline receptor agonists, especially that the effects of moxonidine appear to be more complex than those ascribed to its sympatholytic effects. Taking into consideration receptor tissue distribution, future studies should investigate the **consequences of cardiac imidazoline $I_1$-receptor regulation**, and accordingly, new drugs that specifically target heart imidazoline $I_1$-receptors would be a good addition to treatment regimens.

**Conflict of interest statement**

None.

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**References**


Amann K, Greber D, Gharehbaghi H, Wiest G, Lange B, Ganten U, et al. Effects of nifedipine and moxonidine on cardiac structure in spontaneously hypertensive rats. Stereological studies on myocytes, capillaries, arteries, and cardiac interstitium. *Am J Hypertension* 1992;5:76-83.

Aukrust P, Ueland T, Lien E, Bendtzen K, Müller F, Andreassen AK, et al. Cytokine network in congestive heart failure secondary to ischemic of idiopathic dilated cardiomyopathy. *Am J Cardiol* 1999;83:376-82.

Bajusz E. Hereditary cardiomyopathy: a new disease model. *Am Heart J* 1969;77:686-96.

Bristow MR. Tumor necrosis factor-alpha and cardiomyopathy. Circulation 1998;97:1340-1.

Cheitlin MD, Armstrong WF, Aurigemma GP, Beller GA, Bierman FZ, Davis JL, et al. ACC/AHA/ASE 2003 guideline update for the clinical application of echocardiography--summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol* 2003;42:954-70.

Cohn JN, Pfeffer MA, Rouleau J, Sharpe N, Swedberg K, Straub M, et al.; MOXCON Investigators. Adverse mortality effect of central sympathetic inhibition with sustained-release moxonidine in patients with heart failure (MOXCON). *Eur J Heart Failure* 2003;5:659-67.

Colucci WS, Sawyer DB, Singh K, Communal C. Adrenergic overload and apoptosis in heart failure: implications for therapy. *J Card Failure* 2000;6 (2 Suppl 1):1-7.

Dent MR, Das S, Dhalla NS. Alterations in both death and survival signals for apoptosis in heart failure due to volume overload. *J Mol Cell Cardiol* 2007;43:726-32.

Doesch AO, Mueller S, Konstandin M, Celik S, Kristen A, Frankenstein L, , et al. Effects of protein A immunoadsorption in patients with chronic dilated cardiomyopathy. *J Clin Apher* 2010 Sep 7. [Epub ahead of print]

El-Ayoubi R, Gutkowska J, Regunathan S, Mukaddam-Daher S. Imidazoline Receptors in the Heart: Characterization, Distribution, and Regulation. *J Cardiovasc Pharmacol* 2002;39:875-83.

El-Ayoubi R, Menaouar A, Gutkowska J, Mukaddam-Daher S. Normalization of up-regulated cardiac imidazoline $I_1$-receptors and natriuretic peptides by chronic treatment with moxonidine. *Ann NY Acad Sci* 2003;009:274-8.

Esposito G, Rapacciuolo A, Naga Prasad SV, Takaoka H, Thomas SA, Koch WJ, et al. Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. *Circulation* 2002;105:85-92.

Fedak PW, Verma S, Weisel RD, Li RK. Cardiac remodeling and failure: From molecules to man, Part I. *Cardiovasc Pathol* 2005;14:1-11.

Foo RS, Mani K, Kitsis RN. Death begets failure in the heart. *J Clin Invest* 2005;115:565-71.

Giudice PL, Gagnol JP, Bellucci A, Buffone G, Careddu A, Magni G, et al. Autonomic nervous system activity imbalance in cardiomyopathic hamster. J *Cardiovasc Pharmacol* 2000;36:369-75.

Haxhiu MA, Dreshaj I, Schafer SG, Ernsberger P. Selective antihypertensive action of moxonidine is mediated mainly by I1-imidazoline receptors in the rostral ventrolateral medulla. *J Cardiovasc Pharmacol* 1994;24:S1-8.

Honsho S, Nishikawa S, Amano K, Zen K, Adachi Y, Kishita E, et al. Pressure-mediated hypertrophy and mechanical stretch induces IL-1 release and subsequent IGF-1 generation to maintain compensative hypertrophy by affecting Akt and JNK pathways. *Circ Res* 2009;105:1149-58.

Kabara JJ, Riggin RM, Kissinger PT. Abnormal levels of urinary catecholamines in dystrophic mice and hamsters. *Proc Soc Exp Biol Med* 1976;15:168-72.

Kawada T, Nakatsuru Y, Sakamoto A, Koizumi T, Shin WS, Okai-Matsuo Y, et al. Strain- and age-dependent loss of sarcoglycan complex in cardiomyopathic hamster hearts and its re-expression by delta-sarcoglycan gene transfer in vivo. *FEBS Lett* 1999;458:405–8.

Kawai K, Qin F, Shite J, Mao W, Fukuoka S, Liang CS. Importance of antioxidant and antiapoptotic effects of beta-receptor blockers in heart failure therapy. *Am J Physiol Heart* Circ *Physiol* 2004;287:H1003-12.

Kaye DM, Lefkovits J, Jennings GL, Bergin P, Broughton A, Esler MD. Adverse consequences of high sympathetic nervous activity in the failing human heart. *J Am Coll Cardiol* 1995;26:1257-63.

Kubota T, Miyagishima M, Frye CS, Alber SM, Bounoutas GS, Kadokami T, et al. Overexpression of tumor necrosis factor- alpha activates both anti- and pro-apoptotic pathways in the myocardium. *J Mol* Cell *Cardiol* 2001;33:1331-44.

Kurum T, Tatli E, Yuksel M. Effects of carvedilol on plasma levels of pro-inflammatory cytokines in patients with ischemic and nonischemic dilated cardiomyopathy. Tex *Heart Inst* J 2007;34:52-9.

Leri A, Liu Y, Malhotra A, Li Q, Stiegler P, Claudio PP, et al. Pacing-induced heart failure in dogs enhances the expression of p53 and p53-dependent genes in ventricular myocytes. *Circulation* 1998;97:194-203.

Mayer B, Holmer SR, Hengstenberg C, Lieb W, Pfeifer M, Schunkert H. Functional improvement in heart failure patients treated with beta-blockers is associated with a decline of cytokine levels. *Int J Cardiol* 2005;103:182-6.

Mobini R, Fu M, Jansson PA, Bergh CH, Scharin Täng M, Waagstein F, et al. Influence of central inhibition of sympathetic nervous activity on myocardial metabolism in chronic heart failure: acute effects of the imidazoline I1-receptor agonist moxonidine. *Clin Sci* (Lond) 2006;110:329-36.

Moorjani N, Catarino P, Trabzuni D, Saleh S, Moorji A, Dzimiri N, et al. Upregulation of Bcl-2 proteins during the transition to pressure overload-induced heart failure. *Int J Cardiol* 2007;116:27-33.

Motz W, Scheler S, Mox B. The potential clinical application of moxonidine in congestive heart failure. Rev *Contemp Pharmacother* 1998;9:473-9.

Mukaddam-Daher S, Gutkowska J. Atrial natriuretic peptide is involved in the renal actions of moxonidine. *Hypertension* 2000;35:1215-20.

Mukaddam-Daher S, Menaouar A, Paquette P-A, Jankowski M, Gutkowska J, Gillis M-A, et al. Hemodynamic and cardiac effects of chronic eprosartan and moxonidine therapy in Stroke Prone Spontaneous Hypertensive Rats. *Hypertension* 2009;53:775-81.

Nance DM, Sanders VM. Autonomic innervation and regulation of the immune system (1987-2007). *Brain Behav Immun* 2007;21:736-45.

Oldenburg O, Bitter T, Lehmann R, Korte S, Dimitriadis Z, Faber L, et.al. Adaptive servoventilation improves cardiac function and respiratory stability. *Clin Res Cardiol* Published online 12 Sept. 2010.

Ollivier JP, Christen MO. I1-imidazoline-receptor agonists in the treatment of hypertension: an appraisal of clinical experience. *J Cardiovasc Pharmacol* 1994;24:S39-48.

Paquette PA, Duguay D, El-Ayoubi R, Menaouar A, Danalache B, Gutkowska J, et al. Control of left ventricular mass by moxonidine involves reduced DNA synthesis and enhanced DNA fragmentation. Br J *Pharmacol* 2008;153:459-67.

Pauschinger M, Knopf D, Petschauer S, Doerner A, Poller W, Schwimmbeck PL, et al. Dilated cardiomyopathy is associated with significant changes in collagen type I/III ratio. *Circulation* 1999;99:2750-6.

Petrov VV, Fagard RH, Lijnen PJ. Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension* 2002;39:258-63.

Rauchhaus M, Doehner W, Francis DP, Davos C, Kemp M, Liebenthal C, et al. Plasma cytokine parameters and mortality in patients with chronic heart failure. *Circulation* 2000;102:3060-7.

Reffelmann T, Kloner RA. Trans-thoracic echocardiography in rats. Evaluation of commonly used indices of left ventricular dimensions, contractile performance, and hypertrophy in a genetic model of hypertrophic heart failure (SHHF-Mcc-facp-Rats) in comparison with Wistar rats during aging. *Basic Res Cardiol* 2003;98:275-84.

Siwik DA, Chang DL-F, Colucci WS. Interleukin-1β and tumor necrosis factor-a decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. Circ *Res* 2000;86:1259-65.

Stilli D, Bocchi L, Berni R, Zaniboni M, Cacciani F, Chaponnier C, et al. Alpha-skeletal actin expression, ventricular fibrosis and heart function correlate with the degree of pressure overload cardiac hypertrophy. *Exp Physiol* 2006;91:571-80.

Swedberg K, Bristow MR, Cohn JN, Dargie H, Straub M, Wiltse C, et al. Moxonidine Safety and Efficacy (MOXSE) Investigators. Effects of sustained-release moxonidine, an imidazoline agonist, on plasma norepinephrine in patients with chronic heart failure. *Circulation* 2002;105:1797-803.

Timonen P, Magga J, Risteli J, Punnonen K, Vanninen E, Turpeinen A, et al. Cytokines, interstitial collagen and ventricular remodelling in dilated cardiomyopathy. *Int J Cardiol* 2008;124:293-300.

Van Kerckhoven R, van Veen TA, Boomsma F, Saxena PR, Schoemaker RG. Chronic administration of moxonidine suppresses sympathetic activation in a rat heart failure model. *Eur J Pharmacol* 2000;397:113-20.

Yokoyama T, Vaca L, Rossen RD, Durante W, Hazarika P, Mann DL. Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. *J Clin Invest* 1993;92:2303-12.

Zhang J, Abdel-Rahman AA. Inhibition of nischarin expression attenuates rilmenidine-evoked hypotension and phosphorylated extracellular signal-regulated kinase1/2 production in the rostral ventrolateral medulla of rats. *J Pharmacol Exp Ther* 2008;324:72-8.

## Legends to Figure and Tables

**Fig. 1**: **A.** Representative M-Mode Short axis views showing consecutive cycles of ventricular dimensions, in 6- and 10-month-old hamsters. **B.** Percent FS (Fractional Shortening) and EF (Ejection Fraction) calculated from short axis views. n=6-12 per group. *P<0.01 vs. normal F1B; †P< 0.01 vs. pre-treatment; §p<0.02 vs. vehicle-treated BIO 14.6 cardiomyopathic hamsters. Moxonidine Low (2.4 mg/kg/day); Moxonidine High (9.6 mg/kg/day).

**Fig. 2**: **A.** Photomicrographs of heart cross sections (400x magnification) from BIO 14.6 cardiomyopathic hamsters after 1 month treatment with moxonidine or vehicle. Apoptotic cells are shown as brown nuclei with condensed chromatin. **B.** Bargraphs represent number of apoptotic cells in each group, in comparison to age-matched normal F1B hamsters. n=4 per group. #P<0.01 vs. vehicle-treated BIO 14.6 cardiomyopathic hamsters.

**Fig. 3**: Western blot analysis of the effect of treatments on left ventricular Bax and Bcl2. Bargraphs depict the densitometric measurement of Bax/Bcl2 ratio (normalized to GAPDH, loading control), presented as percent of corresponding normal control (100%). n=6-8 per group. *P<0.01 vs. F1B; ‡P<0.05, #P<0.01 vs. vehicle-treated BIO 14.6 hamsters.

**Fig. 4: A.** Photomicrographs of Masson's Trichrome-stained heart sections (collagen deposition appears in blue) from BIO 14.6 cardiomyopathic hamsters after 1 month treatment with moxonidine, as compared to age-matched normal F1B hamsters. **B.** Bargraphs represent collagen deposition from 5 hamsters, each. *P<0.01 vs. F1B; ‡P<0.05 vs. vehicle-treated BIO 14.6 hamsters. (200x magnification)

**Fig. 5**: Western blot analysis of the effect of treatments on left ventricular collagen I and III. Bargraphs depict the densitometric measurement of collagen I and collagen III and the ratio of collagen I to III, presented as percent of corresponding control (100%). n=6-8 per group. *P<0.01 vs. F1B; #P<0.01 vs. vehicle-treated BIO 14.6 hamsters.

**Fig. 6**: Western blot analysis of the effect of treatments on left ventricular alpha smooth muscle actin (α-SMA). Bargraphs depict the densitometric measurement of α-SMA (normalized to GAPDH, loading control), presented as percent of corresponding normal control (100%). n=6-8 per group. †P<0.05 vs. F1B; §p<0.02 vs. vehicle-treated BIO 14.6 hamsters.

**Fig. 7**: **A**. Effect of treatments on plasma cytokines, IL-1β, TNF-α, IL-6 and IL-10. **B.** Effect of treatments on left ventricular IL-1β and TNF-α. n=6-8 per group. §p<0.02, ‡P<0.05 vs. vehicle-treated BIO 14.6 hamsters.

**Fig. 8**: Western blot analysis of the effect of treatments on left ventricular iNOS, p-NF-κB and total NF-κB. Bargraphs depict the densitometric measurement of the ratio of p-NF-κB to total NF-κB (normalized to GAPDH, loading control), presented as percent of corresponding normal control (100%). n=6-8 per group. §p<0.02 vs. vehicle-treated BIO 14.6 hamsters.

**Fig. 9**: **A.** Representative photomicrographs of the immunohistochemical images (400x magnification) depicting nischarin (imidazoline receptor)-immunoreactive cells in the heart from BIO 14.6 cardiomyopathic hamsters after 1-month treatment with moxonidine, as compared to age-matched normal F1B hamsters. **B.** Bargraphs showing immunoreactivity data obtained from different treatment and control groups. n=5 per group. *P<0.05 vs. F1B; ‡P<0.05 vs. vehicle-treated BIO 14.6 hamsters; $P<0.05 vs. low moxonidine dose.

**Table 1.** Hemodynamic and physical parameters in normal (F1B) hamsters and in 6-month-old cardiomyopathic (BIO 14.6) hamsters after 4 weeks of treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

**Table 2**. Hemodynamic and physical parameters in normal (F1B) hamsters and in 10-month-old cardiomyopathic (BIO 14.6) hamsters after 4 weeks of treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

**Table 3.** Parameters derived from echocardiographic and Doppler assessment of LV function measured in 6-month-old normal F1B hamsters and in cardiomyopathic BIO 14.6 hamsters before (pre-treatment) and 4 weeks after treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

**Table 4.** Parameters derived from echocardiographic and Doppler assessment of LV function measured in 10-month-old normal F1B hamsters and in cardiomyopathic BIO 14.6 hamsters before (pre-treatment) and 4 weeks after treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

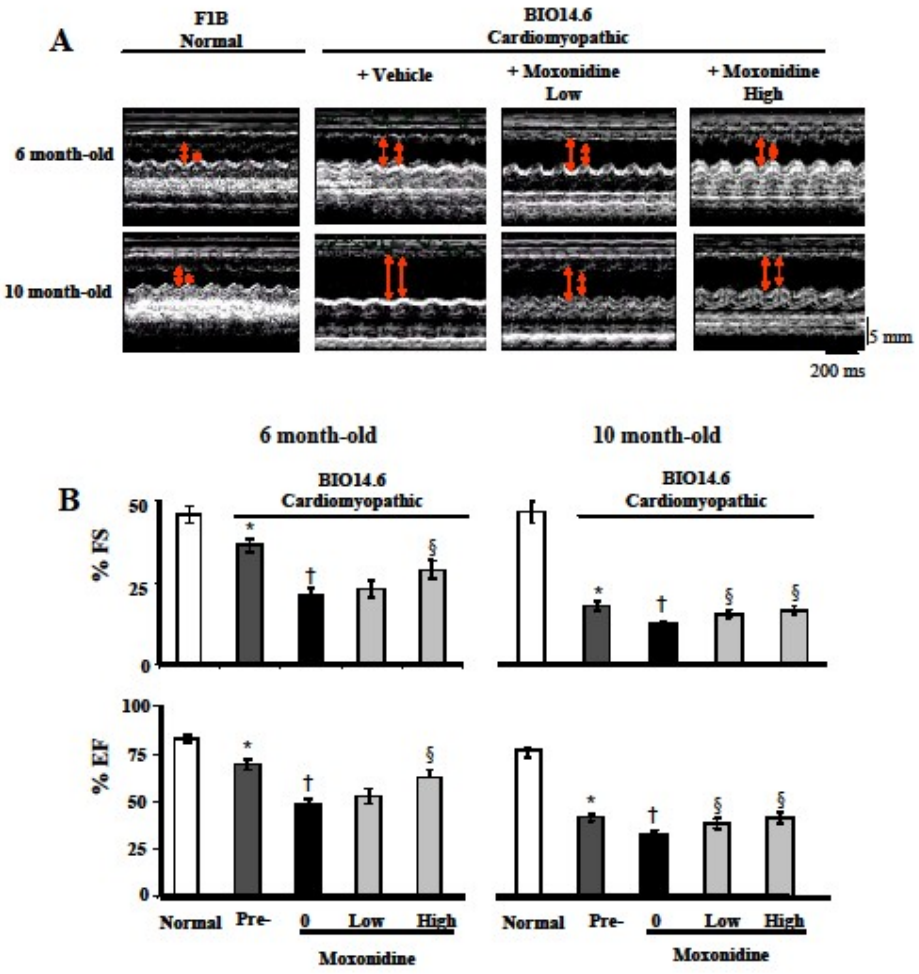


Fig. 1
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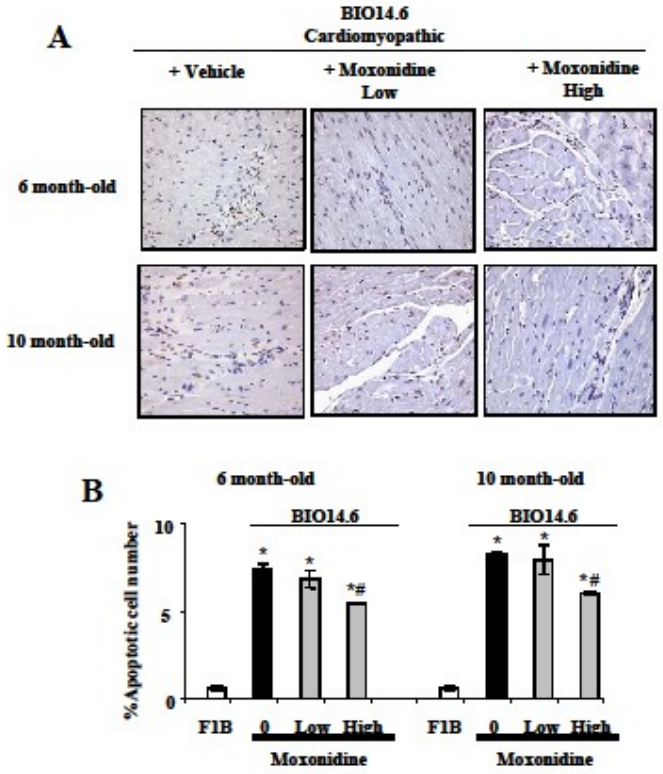
A
BIO14.6
Cardiomyopathic
+ Vehicle
+ Moxonidine Low
+ Moxonidine High
6 month-old
10 month-old
B
6 month-old
10 month-old
BIO14.6
BIO14.6
%Apoptotic cell number
10
5
0
*
*
*#
*
*
*#
F1B
0
Low
High
F1B
0
Low
High
Moxonidine
Moxonidine

Fig. 2
Color

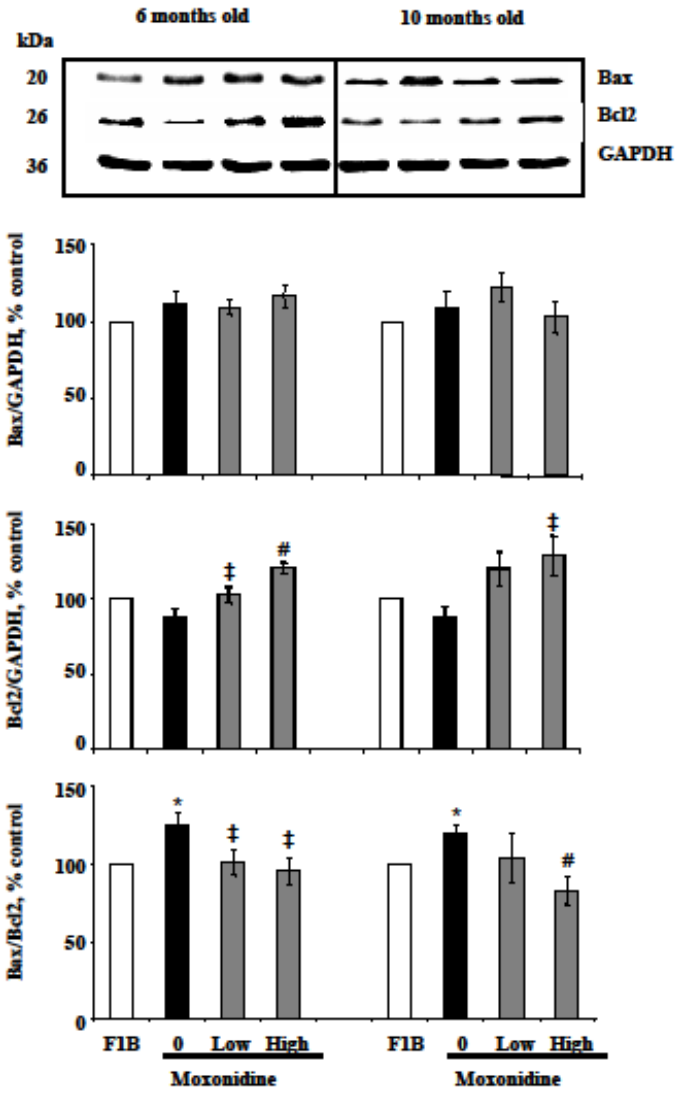
6 months old
10 months old
kDa
20
26
36
Bax
Bcl2
GAPDH
Bax/GAPDH, % control
Bcl2/GAPDH, % control
Bax/Bcl2, % control
150
100
50
0
*
‡
#
F1B
0
Low
High
Moxonidine

Fig. 3:

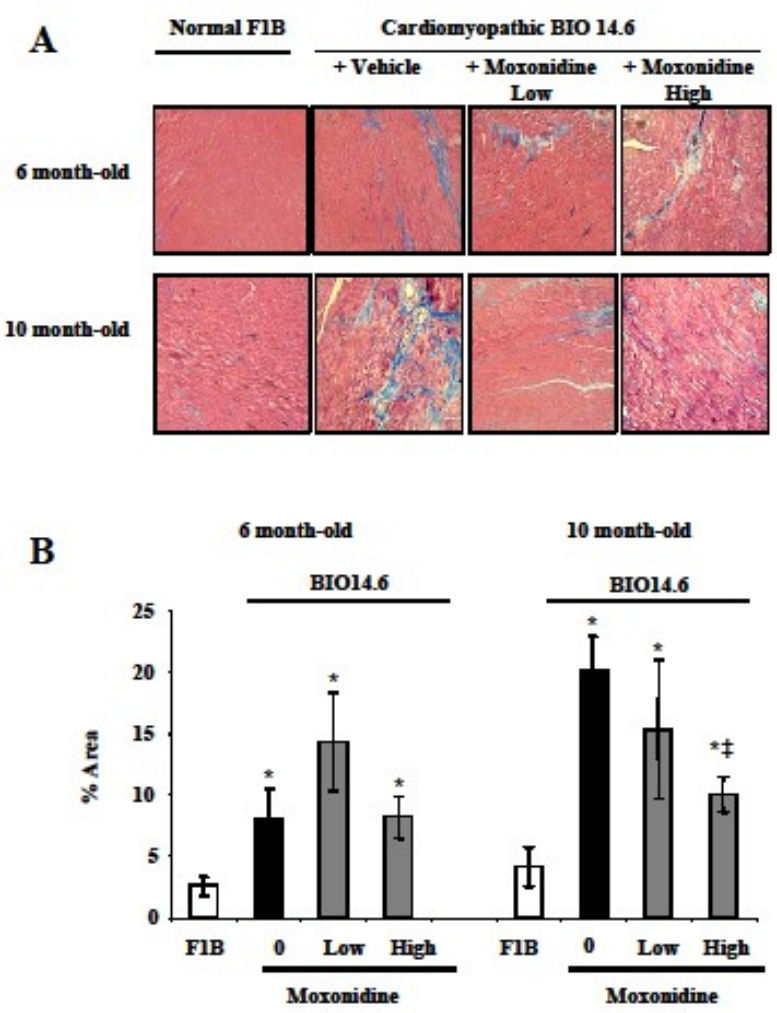
A
Normal F1B
Cardiomyopathic BIO 14.6
+ Vehicle
+ Moxonidine Low
+ Moxonidine High
6 month-old
10 month-old
B
6 month-old
10 month-old
BIO14.6
BIO14.6
% Area
25
20
15
10
5
0
F1B
0
Low
High
F1B
0
Low
High
Moxonidine
Moxonidine

Fig. 4
Color

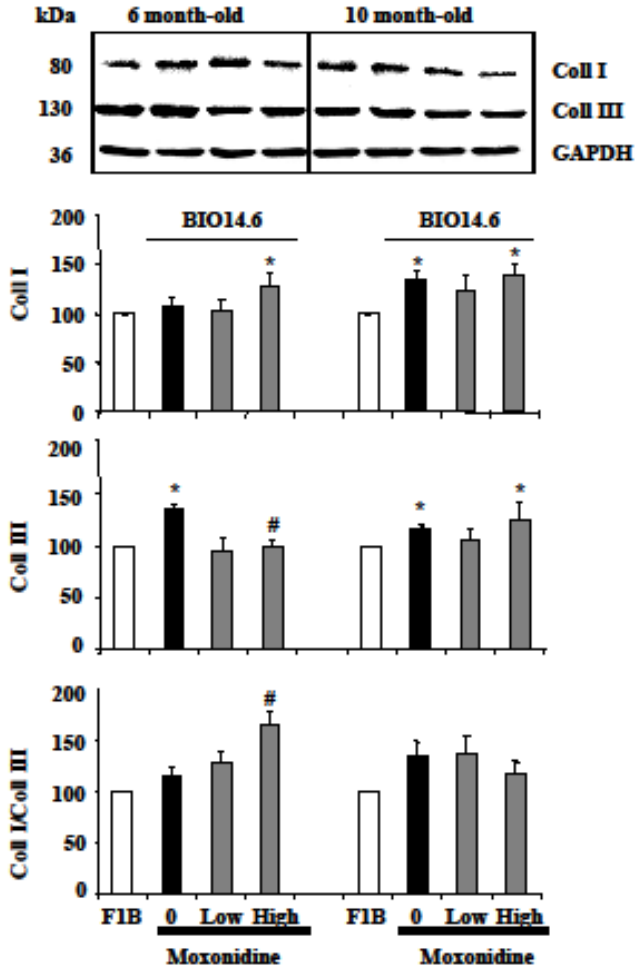
kDa
6 month-old
10 month-old
80
130
36
Coll I
Coll III
GAPDH
BIO14.6
BIO14.6
Coll I
Coll III
Coll I/Coll III
200
150
100
50
0
*
#
F1B
0
Low
High
Moxonidine

Fig. 5

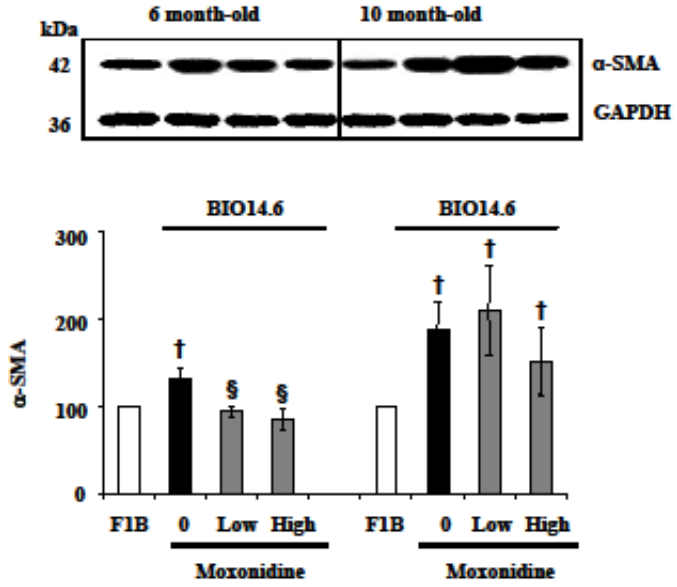
6 month-old
10 month-old
kDa
42
36
α-SMA
GAPDH
BIO14.6
BIO14.6
300
200
100
0
α-SMA
†
§
§
†
†
†
F1B
0
Low
High
F1B
0
Low
High
Moxonidine
Moxonidine

Fig. 6

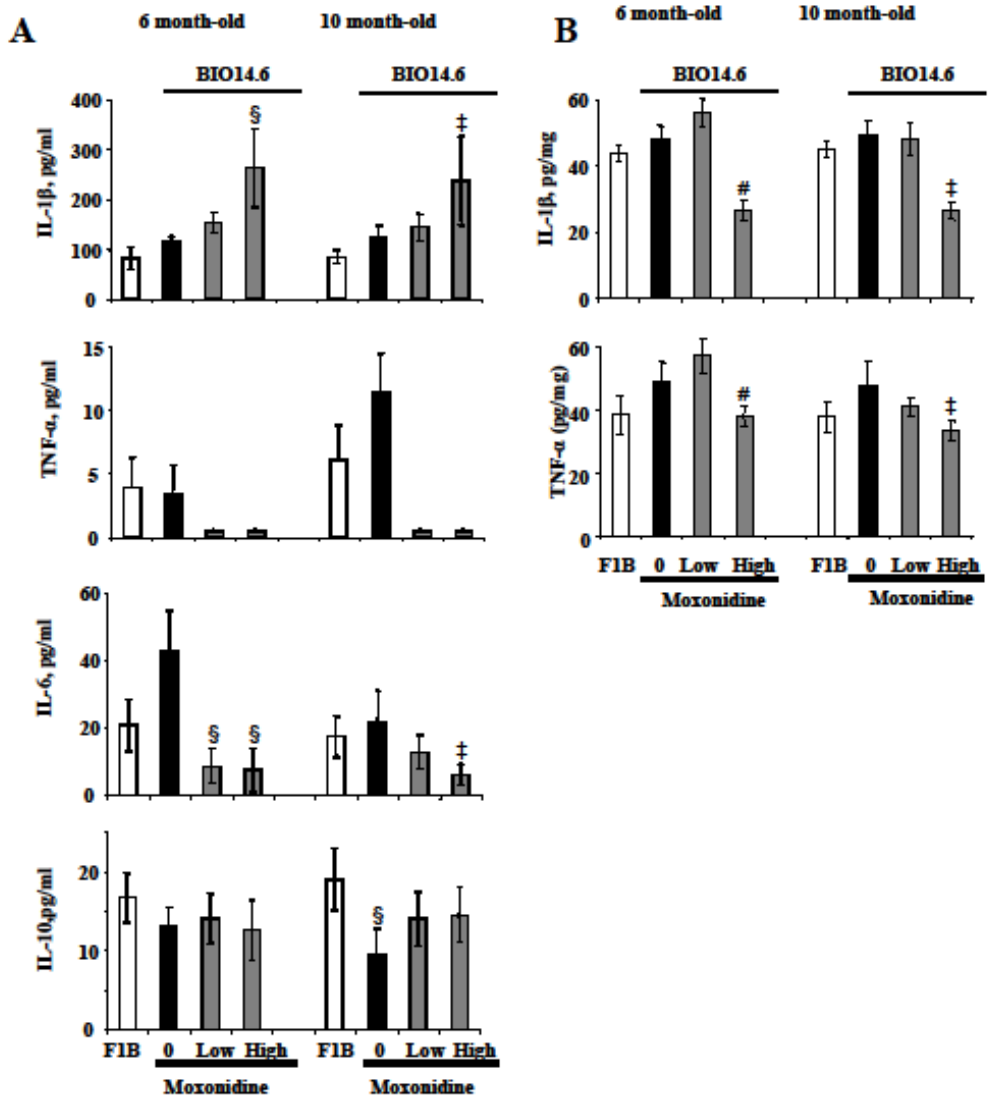
A
6 month-old
10 month-old
BIO14.6
BIO14.6
IL-1β, pg/ml
TNF-α, pg/ml
IL-6, pg/ml
IL-10,pg/ml
F1B
0
Low
High
Moxonidine
B
6 month-old
10 month-old
BIO14.6
BIO14.6
IL-1β, pg/mg
TNF-α (pg/mg)
F1B
0
Low
High
Moxonidine

Fig. 7

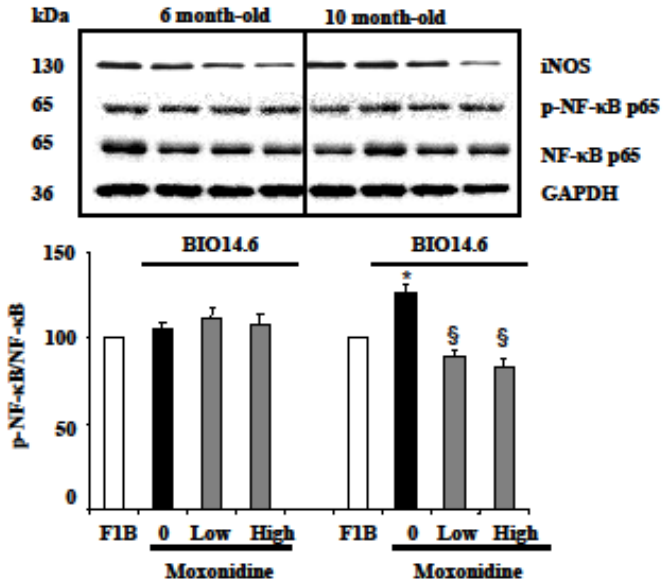
kDa
6 month-old
10 month-old
130
65
65
36
iNOS
p-NF-κB p65
NF-κB p65
GAPDH
BIO14.6
BIO14.6
150
100
50
0
p-NF-κB/NF-κB
*
§
§
F1B
0
Low
High
F1B
0
Low
High
Moxonidine
Moxonidine

Fig. 8

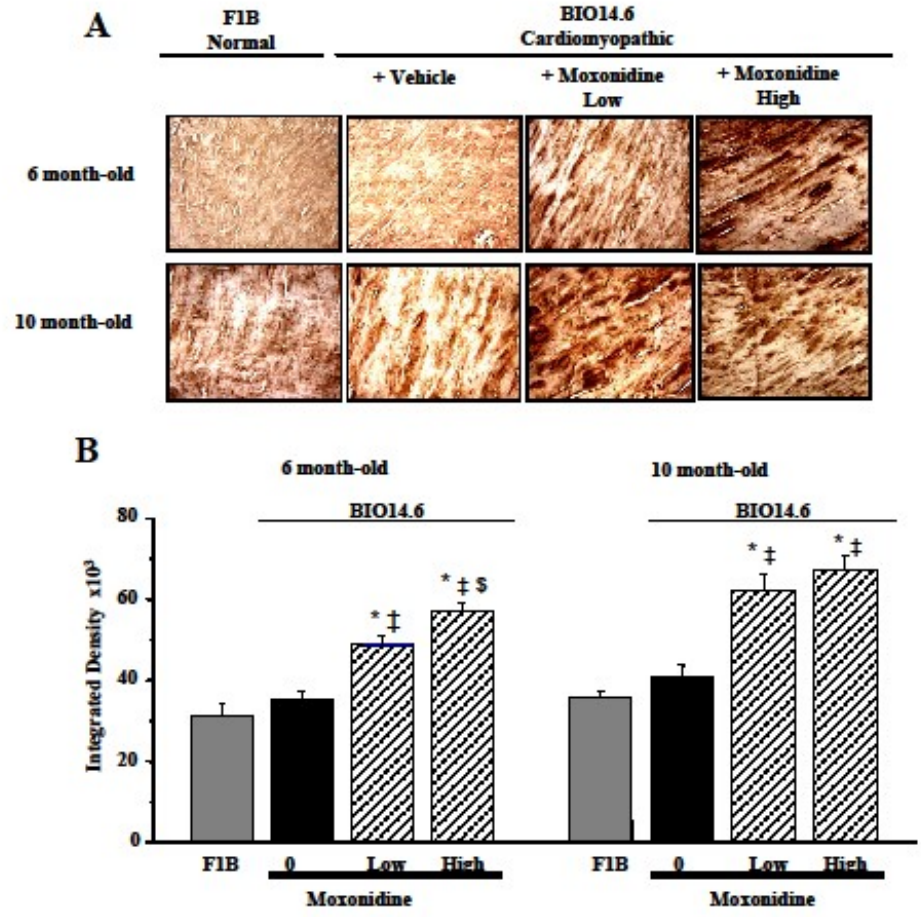
A
F1B
Normal
BIO14.6
Cardiomyopathic
+ Vehicle
+ Moxonidine
Low
+ Moxonidine
High
6 month-old
10 month-old
B
6 month-old
10 month-old
BIO14.6
BIO14.6
Integrated Density x10³
80
60
40
20
0
* ‡
* ‡ $
* ‡
* ‡
F1B
0
Low
High
F1B
0
Low
High
Moxonidine
Moxonidine

Fig. 9
Color

**Table 1.** Hemodynamic and physical parameters in normal (F1B) hamsters and in 6-month-old cardiomyopathic (BIO 14.6) hamsters after 4 weeks of treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

| | F1B | BIO 14.6 | | |
|---|---|---|---|---|
| | | Vehicle | Moxonidine Low | Moxonidine High |
| **Hemodynamic and Physical Parameters** | **n=10** | **n=12** | **n=12** | **n=8** |
| BW, g | 127±3 | 113±3* | 113±4* | 109±3* |
| MAP, mm Hg | 108±3 | 78±4† | 75±4† | 68±4* |
| Lung /BW, mg/g | 0.45±0.01 | 0.42±0.01 | 0.41±0.01 | 0.41±0.01 |
| RA/BW, mg/g | 0.09±0.01 | 0.10±0.01 | 0.10±0.01 | 0.11±0.00 |
| LA/BW, mg/g | 0.17±0.00 | 0.12±0.02 | 0.09±0.01 | 0.10±0.01 |
| RV/BW, mg/g | 0.65±0.03 | 0.75±0.03† | 0.73±0.03† | 0.83±0.06† |
| LVM, mg | 300±22 | 359±8* | 355±12* | 369±16* |
| LVM/tibia length, mg/mm | 8.9±0.7 | 10.7±0.3 | 10.7±0.3 | 11.2±0.5 |
| LVM/BW, mg/g | 2.4±0.1 | 3.1±0.1* | 3.1±0.1* | 3.1±0.2* |
| LV-CSA, $\mu m^2$ | 450±17 | 662±28* | 557±45†‡ | 517±12‡ |

BW, body weight; MAP, mean arterial pressure; RA, right atria; LA, left atria; LVM, left ventricular mass; *P<0.01; †P<0.05 vs. normal F1B; ‡P<0.05 vs. vehicle-treated hamsters.

**Table 2.** Hemodynamic and physical parameters in normal (F1B) hamsters and in 10-month-old cardiomyopathic (BIO 14.6) hamsters after 4 weeks of treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

| | F1B | BIO 14.6 | | |
|---|---|---|---|---|
| | | Vehicle | Moxonidine Low | Moxonidine High |
| **Hemodynamic and Physical Parameters** | **n=6** | **n=12** | **n=12** | **n=6** |
| BW, g | 140±2 | 110±3* | 115±3* | 117±3* |
| MAP, mm Hg | 97±4 | 57±4* | 56±4* | 51±4* |
| Lung /BW, mg/g | 0.48±0.02 | 0.49±0.03 | 0.48±0.02 | 0.44±0.02 |
| RA/BW, mg/g | 0.06±0.00 | 0.21±0.05 | 0.21±0.03 | 0.16±0.02 |
| LA/BW, mg/g | 0.07±0.00 | 0.17±0.03† | 0.2±0.03† | 0.14±0.03 |
| RV/BW, mg/g | 0.67±0.04 | 1.02±0.05* | 0.89±0.06* | 1.06±0.04* |
| LVM, mg | 358±1 | 390±20 | 391±20 | 410±10 |
| LVM/tibia length, mg/mm | 10.4±0.1 | 11.8±0.4* | 11.7±0.4* | 12.4±0.3* |
| LVM/BW, mg/g | 2.6±0.1 | 3.6±0.2* | 3.4±0.1* | 3.5±0.1* |
| LV-CSA, $\mu m^2$ | 463±3 | 733±56* | 588±22‡ | 587±21‡ |

BW, body weight; MAP, mean arterial pressure; RA, right atria; LA, left atria; LVM, left ventricular mass; *P<0.01, †P<0.05 vs. normal F1B; ‡P<0.05 vs. vehicle-treated hamsters.

**Table 3.** Parameters derived from echocardiographic and Doppler assessment of LV function measured in 6-month-old normal F1B hamsters and in cardiomyopathic BIO 14.6 hamsters before (pre-treatment) and 4 weeks after treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

| Parameter | F1B | BIO 14.6 | | | |
|---|---|---|---|---|---|
| | n= 8 | Pre-Treatment | Vehicle | Moxonidine Low | Moxonidine High |
| | | n= 32 | n= 12 | n= 12 | n= 8 |
| Heart Rate, bpm | 336±9 | 336±7 | 356±13 | 349±11 | 301±23‡ |
| EDD, cm | 0.38±0.01 | 0.49±0.02* | 0.57±0.03* | 0.60±0.02* | 0.53±0.02* |
| ESD, cm | 0.21±0.01 | 0.33±0.02* | 0.45±0.03* | 0.46±0.02* | 0.38±0.03* |
| EDV, µl | 184±13 | 439±47* | 792±116* | 796±91* | 472±62*‡ |
| ESV, µl | 29±5 | 144±23* | 413±78* | 354±50* | 203±43*‡ |
| E cm/sec | 70.7±5.3 | 72.6±1.1 | 71.5±1.1 | 71.5±1.1 | 73.8±1.7 |
| DT cm/sec | 51±2 | 51±1 | 48±2 | 50±5 | 54±2 # |
| Dec rate cm/sec2 | 1338±49 | 1393±37 | 1505±36 | 1571±96 | 1303±57‡ |
| LVET, msec | 86±2 | 84±2 | 75±3* | 79±3 | 84±5‡ |
| LVETc | 6.1±0.2 | 6.1±0.1 | 5.5±0.2† | 5.7±0.1 | 5.8±0.1 |
| IVRTc | 1.7±0.1 | 1.9±0.1 | 2.0±0.1 | 1.9±0.1 | 2.0±0.1 |

EDD, end diastolic diameter; ESD, end systolic diameter; EDV, end diastolic volume; ESV, end systolic volume, E: peak E-wave velocity; DT, E wave deceleration time; Dec rate, E wave deceleration rate; LVET, left ventricular ejection time; LVETc, left ventricular ejection time corrected for heart rate; IVRTc, isovolumetric relaxation time corrected for heart rate. *P<0.01 vs. normal F1B; †P<0.02 vs. pre-treatment; ‡P<0.05, #P<0.01 vs. vehicle-treated hamsters.

**Table 4.** Parameters derived from echocardiographic and Doppler assessment of LV function measured in 10-month-old normal F1B hamsters and in cardiomyopathic BIO 14.6 hamsters before (pre-treatment) and 4 weeks after treatment with vehicle or moxonidine at low (2.4 mg/kg/day) or high (9.6 mg/kg/day) concentrations.

| Parameter | F1B | BIO 14.6 | | | |
|---|---|---|---|---|---|
| | | Pre-Treatment | Vehicle | Moxonidine Low | Moxonidine High |
| | n= 8 | n= 30 | n= 12 | n= 12 | n= 6 |
| Heart rate, bpm | 360±11 | 316± 8 | 304±10 | 310±15 | 257±22‡ |
| EDD, cm | 0.38±0.02 | 0.67±0.02* | 0.71±0.02* | 0.72±0.03* | 0.69±0.03* |
| ESD, cm | 0.23±0.02 | 0.56±0.02* | 0.62±0.02* | 0.61±0.03* | 0.58±0.03* |
| EDV, µl | 191±29 | 1118±86* | 1329±111* | 1448±152* | 1252±135* |
| ESV, µl | 45±10 | 648±63* | 861±85* | 858±116* | 717±101* |
| E cm/sec | 70.7±3.2 | 75.3±1.1 | 70.7±3.7 | 74.6±.4 | 79.0±0.2 |
| DT cm/sec | 47±1 | 45±1 | 44±3 | 40±2 | 56±5# |
| Dec rate cm/sec2 | 1391±34 | 1593±53 | 1763±133 | 1810±101 | 1289±135‡ |
| LVET, msec | 79±2 | 76±1 | 72±4 | 73±3 | 87±5‡ |
| LVETc | 5.4±0.3 | 5.7±0.1 | 5.1±0.2* | 5.2±0.2 | 5.6±0.1 |
| IVRTc | 1.6±0.1 | 2.3±0.0 | 2.6±0.1 | 2.4±0.1 | 2.3±0.3 |

EDD, end diastolic diameter; ESD, end systolic diameter; EDV, end diastolic volume; ESV, end systolic volume, E: peak E-wave velocity; DT, E wave deceleration time; Dec rate, E wave deceleration rate; LVET, left ventricular ejection time; LVETc, left ventricular ejection time corrected for heart rate; IVRTc, isovolumetric relaxation time corrected for heart rate. *P<0.01 vs. F1B; #P<0.01, ‡P<0.05 vs. vehicle-treated hamsters.

## 3.4 Article 3. Nischarin over-expression opposes cell-death induced by oxidative stress

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# Nischarin Over-Expression Opposes Cell-Death Induced By Oxidative Stress

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## Abstract

Nischarin (murine imidazoline $I_1$-receptor) has been implicated in central regulation of blood pressure and cardiovascular control. Previous studies have also shown that nischarin associates with α5 integrin and that nischarin over-expression in a fibroblastic cell line inhibits cell motility through inhibition of integrin α5-Rac1 signalling. Rac1 is a small G protein whose activation is linked to increased generation of reactive oxygen species (ROS) and subsequent cell hypertrophy, proliferation and apoptosis. The association of nischarin to Rac1 prompted us to investigate in human embryonic kidney (HEK293) cells, the role of nischarin in cell survival in response to oxidative stress and the signalling proteins, MAPKs and Akt (PKB), implicated in cell death and survival. HEK293 cells were transiently transfected with full length GFP-tagged nischarin cDNA or empty vector (control), using lipofectamine 2000 reagent. 48 h post-transfection, cells were plated in 96-or 6-well plates for 24 h and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). After inducing quiescence in DMEM+0.1%FBS for 12 h, cells were incubated with treatments associated with oxidative stress (cytokines, LPS, H2O2). $I_1$-receptor mediated effects were evaluated by co-incubation with a selective $I_1$-receptor agonist, moxonidine ($10^{-7}$ and $10^{-5}$ M) with and without imidazoline $I_1$-receptor antagonist, AGN192403 ($10^{-5}$ M). Cell viability was measured after 48 h incubation, by MTT reduction assay. Total and phosphorylated intracellular proteins and nischarin expression were measured by Western blot. Data are presented as mean±SEM of n=9-12, from 2-3 independent cultures. The results show that compared with control cells (100%), nischarin over-expression (5-fold increase) did not modify basal cell viability but increased cell viability in response to ($10^{-7}$ M) moxonidine

(117±2%; P<0.01), effect reversed by co-incubation with AGN192403. Western blot analysis revealed that nischarin transfected cells incubated in DMEM alone had higher ERK1/2 (151%±13, p<0.01) and JNK (121%±6, p<0.01) phosphorylation and that 15 min incubation with moxonidine abolished this increase. Incubation with TNF+IL1β, LPS, or H2O2 resulted in significant (P<0.01) reduction in cell survival, where H2O2 effects were associated with reduced Akt phosphorylation and TNF+IL1β with enhanced MAPKs activities, all in control cells; effects prevented in nischarin-transfected cells. These results show that nischarin over-expression potentiates cell viability in response to moxonidine and opposes cell-death induced by oxidative stress by differential actions on Akt and MAPKs. These studies point to over-expression of nischarin in the heart as a therapeutic tool opposing oxidative stress and promoting cardiac cell survival, a function especially important in heart failure.

Keywords: nischarin, imidazoline receptors, moxonidine, Akt, MAPK, oxidative stress, cell viability, HEK293 cells

## Introduction

The hypotensive action of imidazoline compounds, such as clonidine and moxonidine, is selectively mediated by imidazoline $I_1$-receptors (also known as nischarin), non-adrenergic neurotransmitter receptors primarily present in brainstem medulla and involves activation of ERK, nitric oxide, and cardiac natriuretic peptides, ANP and BNP ([reviewed in [1]). Molecular characterization revealed that $I_1$-receptor/nischarin is not a classical transmembrane receptor; it is rather a protein localized to the cytosol, anchored on the inner layer of plasma membranes. In addition to imidazoline binding, $I_1$-receptor/nischarin associates with α5 integrin and insulin receptor substrate IRS-4 [2], [3], [4], which allows regulation of different biological functions, beyond blood pressure control. Studies by Alahari and colleagues [2], [3], revealed that nischarin over-expression in a fibroblastic cell line inhibits cell motility through inhibition of integrin α5-Rac1 signalling, mediated by PAK-dependent and PAK-independent mechanisms [2], [3]. Rac1 is a small GTPase whose activation is linked to increased generation of reactive oxygen species (ROS) and subsequent cell hypertrophy, proliferation, and apoptosis, through activation of JNK and p38 MAPK [5]. The association of nischarin with Rac1 prompted us to investigate the role of nischarin in cell survival in response to oxidative stress. The signalling proteins, MAPKs and Akt (PKB), implicated in cell death and survival as well as in $I_1$-receptor/nischarin signalling were also investigated.

## Methodology

*Microscopy*

HeLa cells were seeded on polylysine-coated coverslips that were placed in six-well plates and transfected with full length GFP-tagged nischarin cDNA or GFP-tagged empty vector (control) [2],[3], using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. 48 h post-transfection, cells were fixed, permeabilized, then labeled with either mouse anti-GFP antibody and AlexaFluor 488 anti-mouse antibody, or rabbit anti-nischarin antibody. Nuclei were stained with Hoechst. Slides were observed using a Zeiss Axioplan 2 Imaging microscope.

*PCR*

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco Modified Eagle's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 5 mg/ml penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37° C. HEK293 cells, transfected as above, were grown in DMEM+10%FBS for 2 days, then harvested for detection of nischarin mRNA by PCR.

*Measurement of Cell Death and Viability by FACS and MTT*

HEK293 transfected cells were incubated in DMEM supplemented with either 0.1% FBS (serum-deplete) or 10% FBS (serum-replete). In both conditions, cells were incubated without or with $I_1$-receptor/nischarin agonist, moxonidine ($10^{-7}$ &$10^{-5}$ M) and $I_1$-receptor antagonist, AGN192403 (AGN, $10^{-5}$ M), or mixed $I_1$-receptor/$\alpha$2-adrenoceptor antagonist, efaroxan ($10^{-5}$ M). After 24 h, cells were stained with propidium iodide (PI). PI positive cells, reflecting cell death, were counted on 10,000 cells, using a LSR II flow cytometer. In addition, cells plated in 96-well plates were incubated without and with $10^{-7}$ &$10^{-5}$ M moxonidine and $10^{-5}$ M AGN. Other cells were subjected to oxidative stress by incubation either with 10 ng/ml TNF-$\alpha$, 5 ng/ml IL-1$\beta$, TNF+IL1$\beta$, 100 ng/ml lipopolysccharide (LPS), or $10^{-4}$ M hydrogen peroxide (H2O2). Viable cells were detected after 48 h, by incubation for 4 h at 37°C with tetrazolium salt (MTT). Metabolically oxidized formazan product, reflecting mitochondrial activity/viability, was read from an absorbance plate reader at 570 nm.

*Western Blot*

Signaling proteins involved in cell growth and death were evaluated by western blot, in cells exposed to different treatments for 15 min, or 1 or 24 h. The antibodies used were (1:1000) rabbit polyclonal phospho-specific Akt-1 (Ser473), extracellular signal-regulated kinase (P-ERK1/2, Thr202/Tyr204), p38 mitogen-activated protein kinase (P-p38 MAPK, Thr180/Tyr182), and c-Jun N-terminal kinase (P-JNK, Thr183-Tyr185), and antibodies against inducible nitric oxide synthase (iNOS) and nischarin. Loading was assessed by anti-GAPDH or anti-$\beta$-actin.

*Statistical Analysis*

Data were compared to corresponding DMEM-incubated cells (considered as 100%) and presented as mean±S.E.M. Statistical significance was evaluated using unpaired Student's t-test. $p<0.05$ was considered significant.

## Results

*Detection of Nischarin Expression*

Nischarin transfection in HeLa cells detected by fluorescent microscopy reveals that nischarin (which was undetectable in native cells) is diffused in cell cytosol, but not in nuclei (Fig.1). In addition, PCR and Western blotting reveal that nischarin is expressed at very low levels in HEK293 cells but transfection results in significantly increased nischarin mRNA as well as 5 fold increased nischarin protein (not shown).

*Effect of Nischarin Expression on Cell Death and Viability/Proliferation*

Cell death and viability/proliferation were measured by flow cytometry and MTT assays, respectively, in serum-starved and serum-replete conditions. In serum-starved conditions, only efaroxan and efaroxan+moxonidine caused an increase in cell death (PI+ve) in control cells, whereas AGN had no effect. In nischarin-cells, incubation with AGN or moxonidine, significantly increased cell death, but in combination, cell death was significantly reduced. Also, incubation of nischarin-cells with efaroxan alone had no effect, but efaroxan tended to attenuate moxonidine-induced cell death (Fig.2). There were no significant changes by all treatments in serum-replete conditions (data not shown).

Cell viability/proliferation measured by MTT in serum-starved cells was not affected by moxonidine and AGN or efaroxan (data not shown). However, in serum-replete conditions (Fig. 3, left), cell viability was modestly improved (117±2%, $P<0.01$) in nischarin-transfected cells by incubation with $10^{-7}$ M moxonidine, in comparison to their corresponding DMEM-incubated control cells and to moxonidine-incubated empty vector-transfected cells. The effect was prevented by co-incubation with AGN, indicating an $I_1$-receptor-mediated survival effect of moxonidine.

Fig. 3 (right) shows that exposure of control cells to combined cytokines, LPS, or H2O2 significantly reduced the number of viable cells compared to DMEM control. However, exposure of nischarin-transfected cells to these oxidants resulted in modest but significant increases in cell viability in comparison to corresponding DMEM and empty vector controls. In addition, (data not shown) co-incubation of nischarin-transfected cells with $10^{-7}$ M moxonidine potentiated interleukin-improved cell viability but opposed TNF-improved cell viability, without altering the effects of combined TNF+IL1β, LPS, or H2O2. These results are consistent with an inherent action of nischarin against oxidative stress-reduced cell viability and reveal a stimulus-specific effect of moxonidine.

*Signalling Proteins*

Western blot analysis revealed that control HEK cells contained P-Akt as well as low levels of P-ERK, P-JNK, and P-p38 MAPK. Nischarin expression did not affect basal Akt and p38 phosphorylation but increased ERK (151%±13, $p<0.01$) and JNK (121%±6, $p<0.01$) phosphorylation. To rule out the influence of altered basal values and selectively investigate the effect of moxonidine on Akt and MAPKs, results were compared to corresponding DMEM-incubated cells. Fig. 4 shows that incubation with moxonidine increases p38 and JNK phosphorylation in control cells but not in nischarin-cells. Also, $10^{-7}$ M moxonidine inhibits ERK phosphorylation in nischarin-cells and $10^{-5}$ M moxonidine inhibits ERK phosphorylation in both, nischarin- and control cells (Fig. 4). These results are consistent with anti-apoptotic and anti-inflammatory actions of moxonidine in nischarin-expressing cells.

The effect of nischarin expression on signaling proteins was also evaluated in response to H2O2 and TNF+IL1β, stimuli associated with ROS generation and oxidative stress (Fig. 5). The results show that H2O2 reduces Akt phosphorylation in control cells, but not in nischarin-cells, where Akt phosphorylation is rather significantly increased. On the other hand, H2O2 increases ERK and JNK and reduces p38 phosphorylation in both nischarin- and control cells. Co-incubation with moxonidine does not significantly affect these changes. Incubation with combined TNF+IL1β does not affect Akt phosphorylation but increases ERK, p38, and JNK phosphorylation in control cells. Increased MAPKs are significantly attenuated or prevented in nischarin-cells and not affected by moxonidine (Fig. 5). These results reflect a stimulus-dependent signaling of nischarin.

Inducible NOS can be expressed in many cell types in response to oxidative agents, such as LPS, cytokines, or H2O2, and involves the MAPK cascades [6]. Therefore, we investigated the influence of nischarin transfection and moxonidine on iNOS expression in HEK cells exposed to oxidative stress. Basal iNOS expression was not altered by nischarin transfection. Moxonidine tended to increase iNOS in control cells but significantly inhibited it in nischarin-cells. The effect of $10^{-5}$ M moxonidine was significantly reversed by co-incubation with AGN. Incubation with H2O2 resulted in increased iNOS expression, an effect prevented by moxonidine, only in control cells. In nischarin-cells H2O2-induced iNOS was significantly lower than control and not affected by moxonidine (Fig. 6). Thus, together with low expression of nischarin in control cells, the anti-inflammatory effect of moxonidine does not appear to be totally mediated by $I_1$-receptor/nischarin.

## Conclusions

This study shows that nischarin overexpression results in increased cell viability/proliferation in response to moxonidine, which outweighs increased percent cell death. The overall survival response is associated with moxonidine's inhibition of ERK, JNK, and p38 MAPK, but not Akt activation. Nischarin also opposes the reduced cell viability in response to oxidative stimuli, with differential responses to moxonidine.

Previous studies have shown that $I_1$-receptor/nischarin activation by $I_1$-receptor ligands and/or over-expression increases neuronal PC12 cell survival [7], [8]. Also, activation of $I_1$-receptor/nischarin by moxonidine protects against norepinephrine-induced cardiomyocyte mortality and fibroblast proliferation [9]. The present studies extend to show that nischarin opposes H2O2-induced cell-death and reduced Akt activation. Also, nischarin opposes TNF+IL1β-induced cell death and ERK, JNK, and p38 MAPK activation. This suggests that the control of cell fate by nischarin is stimulus-specific and may be due to the balance between signaling proteins involved in cell proliferation and those involved cell death. The preferential interaction between nischarin and the Akt signaling pathway downstream of H2O2 and the MAPK pathways downstream of cytokines, is consistent with studies showing that Akt provides resistance against H2O2-induced oxidative stress and apoptosis [10] and cytokine-

induced oxidative stress and apoptosis are mediated through downstream signalling of p38 MAPK and JNK [11]. However, the differential signaling between H2O2 and TNF+IL1β may have been influenced by different incubation times (1 h vs. 15 min) and the kinetics of MAPKs and Akt phosphorylation. ERK activation is rapid, peaking at 15 to 30 min and decreases thereafter, while Akt activation remains up to 2 hours. [12]. Alternatively, signalling may differ in response to exogenously applied H2O2 and subsequent ROS generation in comparison to that endogenously produced by cytokines, in terms of concentration and mode of action.

Previous studies in PC12 have shown that $I_1$-receptor agonists cause concentration-dependent increased phosphorylation of ERK1/2, JNK, and Akt [13], [14]. In contrast, the present studies show that incubation of nischarin-expressing HEK293 cells with moxonidine does not affect Akt, but inhibits ERK, JNK, and p38 MAPK activation, albeit at different concentrations. Furthermore, the effect of moxonidine was more pronounced in control HEK293 cells, which express physiologically irrelevant nischarin level. Thus, the effects of moxonidine appear to depend on the cell-type and cellular context, including the level of nischarin expression and resulting imidazoline binding sites as well as proteins that associate with nischarin and downstream signaling pathways. Different cellular context may include measurable basal ERK activity in PC12 [12] but very low in HEK293 cells [4]; overexpression of hIRAS (human nischarin) results in imidazoline binding sites in PC12 but not in HEK293 cells; and hIRAS expression in HEK293 cells has no effect on basal Akt and ERK activation or insulin activation of Akt, but enhances insulin activation of ERK1/2 through association with IRS-4, abundant in HEK cells [4]. We have shown high nischarin mRNA and protein expression in nischarin transfected cells, but must also evaluate resulting imidazoline binding sites. Further studies are also required to explain the differential effects of moxonidine as well as the influence of oxidative stress on proteins that associate with nischarin, such as integrin α5 and IRSs, and others not yet identified. However, the present studies reveal that nischarin over-expression opposes cell-death induced by oxidative stress and point to nischarin overexpression in the heart as a therapeutic target, especially in heart failure, where cardiomyocyte survival is of ultimate importance.

## REFERENCES


[1] Mukaddam-Daher, S. (2012). An "I" on Cardiac Hypertrophic Remodelling: Imidazoline Receptors and Heart Disease. Can J Cardiol. Apr 5. [Epub ahead of print].

[2] Alahari, S.K., Lee, J.W., Juliano, R.L. (2000). Nischarin, a novel protein that interacts with the integrin alpha5 subunit and inhibits cell migration. J Cell Biol 151, 1141-54.

[3] Baranwal, S., Wang, Y., Rathinam, R., et al. (2011). Molecular characterization of the tumor-suppressive function of nischarin in breast cancer. J Natl Cancer Inst 103, 1513-28.

[4] Sano, H., Liu, S.C., Lane, W.S., Piletz, J.E., Lienhard, G.E. (2002). Insulin receptor substrate 4 associates with the protein IRAS. J Biol Chem 277, 19439–47.

[5] Coso, O.A., Chiariello, M., Yu, J.C., et al. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 81(7),1137-46.

[6] Bhat, N.R., Feinstein. D.L., Shen, Q., Bhat, A.N. (2002). p38 MAPK-mediated transcriptional activation of inducible nitric-oxide synthase in glial cells. Roles of nuclear factors, nuclear factor kappa B, cAMP response element-binding protein, CCAAT/enhancer-binding protein-beta, and activating transcription factor-2. J Biol Chem 277(33), 29584-92.

[7] Dontenwill, M., Piletz, J.E., Chen, M., et al. (2003) IRAS is an anti-apoptotic protein. Ann N Y Acad Sci 1009, 400-12.

[8] Dupuy, L., Urosevic, D., Greney, H., Dontenwill, M., Bousquet, P. (2004). I1-imidazoline receptor-mediated effects on apoptotic processes in PC12 cells. Cell Death Differ 11, 1049-52.

[9] Aceros, H., Farah, G., Cobos-Puc, L., Stabile, A., Noiseux, N., Mukaddam-Daher, S. (2011). Improved cardiac structure and performance by moxonidine in SHR involve inhibition of cytokines, p38 MAPK, and Akt. Br J Pharmacol 164, 946–57.

[10] Lu, Y., Zhou, J., Xu, C., et al. (2008) JAK/STAT and PI3K/AKT pathways form a mutual transactivation loop and afford resistance to oxidative stress-induced apoptosis in cardiomyocytes. Cell Physiol Biochem 21, 305–14.

[11] Dhingra, S., Sharma, A.K., Singla, D.K., Singal, P.K. (2007). p38 and ERK1/2 MAPKs mediate the interplay of TNF-alpha and IL-10 in regulating oxidative stress and cardiacmyocyte apoptosis. Am J Physiol Heart Circ Physiol 293, H3524-31.

[12] Andreucci, M., Fuiano, G., Presta, P., et al. (2009). Downregulation of cell survival signalling pathways and increased cell damage in hydrogen peroxide-treated human renal proximal tubular cells by alpha-erythropoietin. Cell Prolif 42(4), 554-61.

[13] Edwards, L., Fishman, D., Horowitz P, Bourbon N, Kester M, Ernsberger P (2001). The I1-imidazoline receptor in PC12 pheochromocytoma cells activates protein kinases C, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK). J Neurochem 79(5), 931-40.

[14] Tesfai, J., Crane, L., Baziard-Mouysset, G., Kennedy, W., Edwards, L.P. (2011). Novel I1-imidazoline S43126 enhance insulin action in PC12 cells. Pharmacol Rep 63(6), 1442-9.

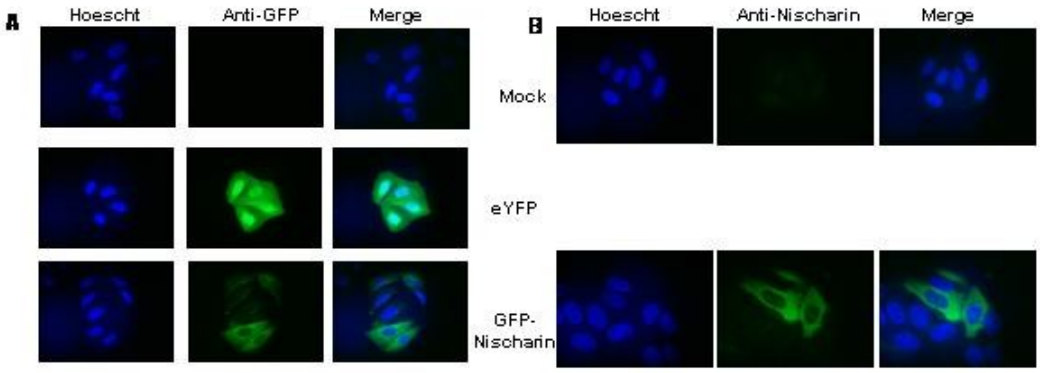


**Fig. 1** Nischarin Over-expression in HeLa cells reveals that Nischarin is diffused in cell cytosol, but not in nuclei. Detection was achieved using 2 different antibodies: A) antiGFP; B) anti-nischarin. Blue = cell nuclei; Green = GFP-nischarin

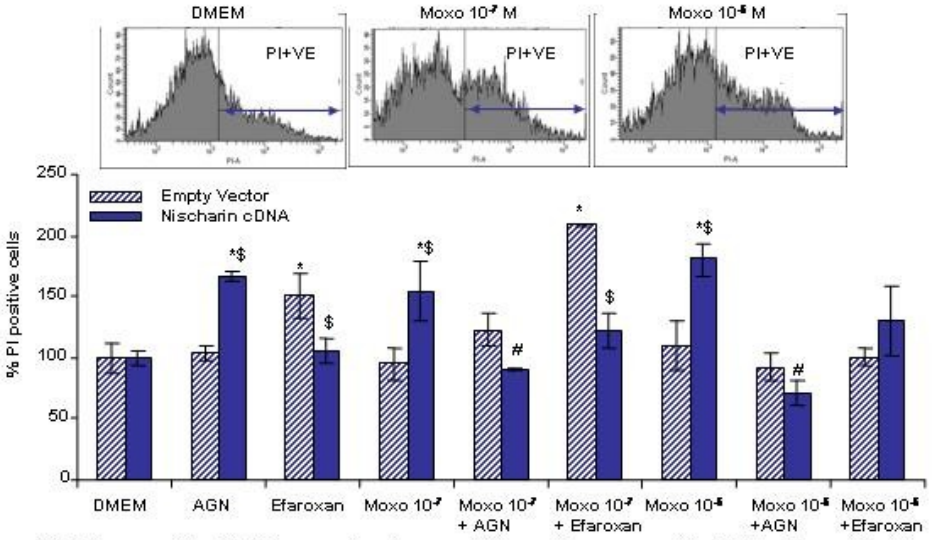


**Fig. 2** Representative FACS images showing percent PI+ve cell measurement by FACS. Bargraph includes results from cells incubated with moxonidine and/or AGN or efaroxan for 24 h.*P<0.01 vs. corresponding DMEM; #P<0.01 vs. corresponding moxonidine; $P<0.01 vs. corresponding empty vector; n=6-9, from 3 independent cultures.

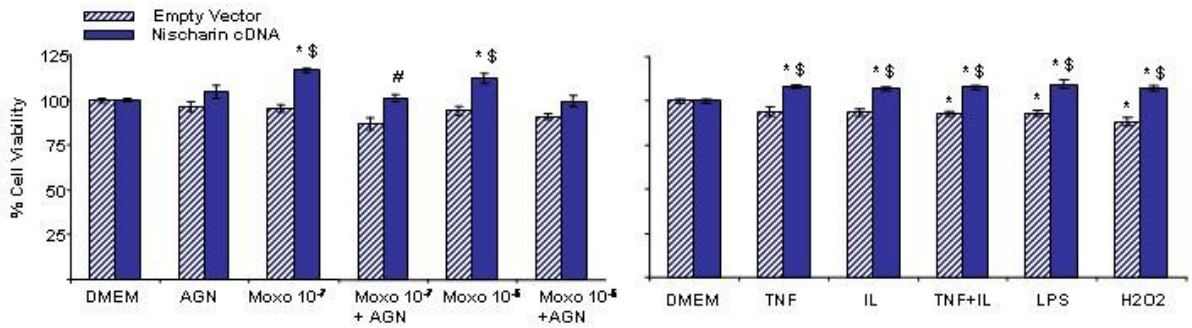


**Fig. 3** MTT measurement of cell viability in HEK293 cells transfected with nischarin or empty vector (control). Cells were incubated with moxonidine ($10^{-7}$ and $10^{-5}$ M) without and with AGN192403 or TNF (10 ng/ml), IL1β (5 ng/ml), TNF+IL1β, LPS (100 ng/ml), or H2O2 ($10^{-4}$ M). *P<0.01 vs. corresponding DMEM; #P<0.01 vs. corresponding moxonidine; $P<0.01 vs. corresponding empty vector; n=12-15, from 3 independent cultures.

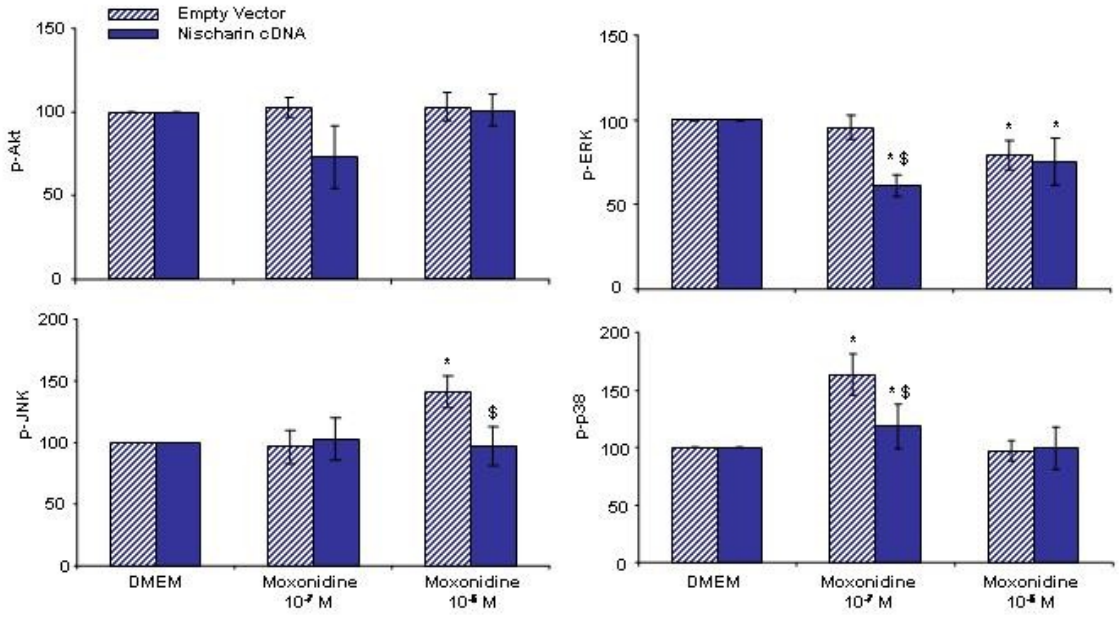


**Fig. 4** Western blot analysis of phosphorylated Akt, ERK, JNK, and p38 MAPK in empty vector and nischarin-transfected cells incubated for 15 min with moxonidine ($10^{-7}$ and $10^{-5}$ M). n=6-9 each from 2 independent cultures. *P<0.01 vs. corresponding DMEM; $P<0.05 vs. corresponding empty-vector-transfected cells.

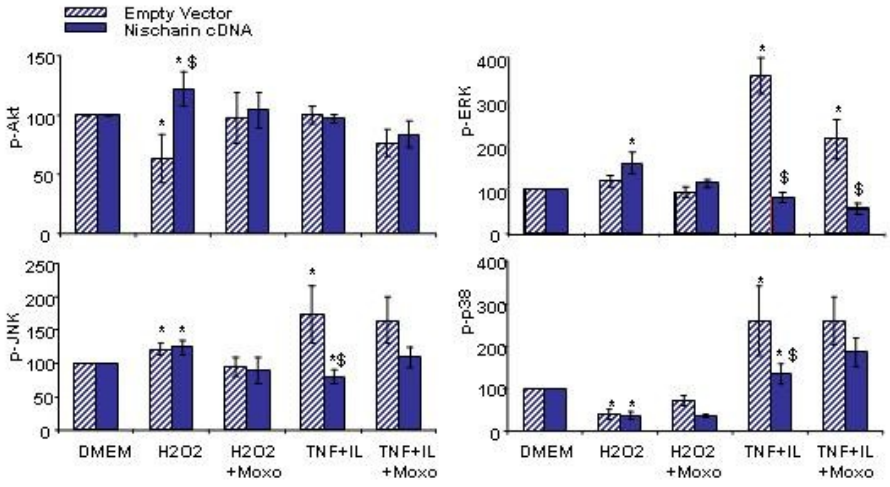


**Fig. 5** Western blot analysis of phosphorylated Akt, ERK1/2, JNK, and p38 MAPK on empty vector- or nischarin-transfected HEK cells after 1h ($10^{-4}$ M H2O2) or 15 min incubation (TNF+IL1β) with or without moxonidine ($10^{-5}$ M). n=6-9 each, from 2 independent cultures. *P<0.01 vs. corresponding DMEM; $P<0.05 vs. corresponding empty-vector-transfected cells.

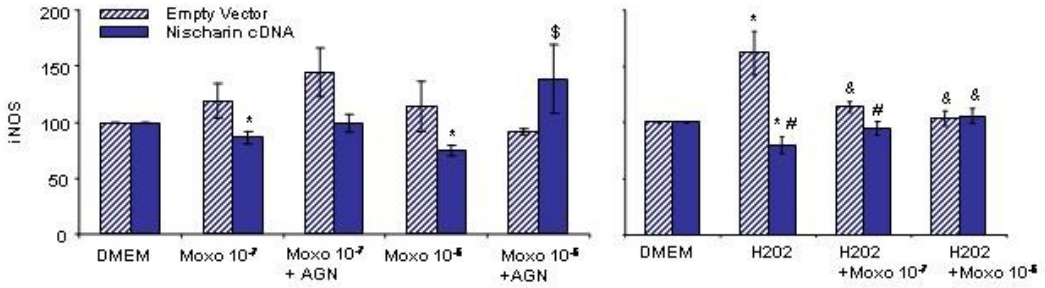


**Fig. 6** Western blot analysis of iNOS protein expression in empty vector- or nischarin-transfected HEK cells. Left, cells were incubated with moxonidine ($10^{-7}$ and $10^{-5}$ M) without and with AGN192403 for 24 h. Right, cells were incubated with moxonidine for 24 h then $10^{-4}$ M $H_2O_2$ was added for 1h. n=6-9 each, from 3 independent; *P<0.01 vs. corresponding DMEM; $P<0.05 vs. corresponding $H_2O_2$+moxonidine; #P<0.01 vs. corresponding empty vector cells; &P<0.02 vs. corresponding $H_2O_2$-incubated cells.

## 3.5 Article 4. Moxonidine modulates cytokine signalling and effects on cardiac cell viability

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# Moxonidine modulates cytokine signalling and effects on cardiac cell viability

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## Abstract

Regression of left ventricular hypertrophy and improved cardiac function in SHR by the centrally acting imidazoline $I_1$-receptor agonist, moxonidine, are associated with differential actions on circulating and cardiac cytokines. Herein, we investigated cell-type specific $I_1$-receptor (also known as nischarin) signalling and the mechanisms through which moxonidine may interfere with cytokines to affect cardiac cell viability. Studies were performed on neonatal rat cardiomyocytes and fibroblasts incubated with interleukin (IL)-1β (5 ng/ml), tumor necrosis factor (TNF)-α (10 ng/ml), and moxonidine ($10^{-7}$ and $10^{-5}$ M), separately and in combination, for 15 min, and 24 and 48 h for the measurement of MAPKs (ERK1/2, JNK, and p38) and Akt activation and inducible NOS (iNOS) expression, by Western blotting, and cardiac cell viability/proliferation and apoptosis by flow cytometry, MTT assay, and Live/Dead assay. Participation of imidazoline $I_1$-receptors and the signalling proteins in the detected effects was identified using imidazoline $I_1$-receptor antagonist and signalling protein inhibitors. The results show that IL-1β, and to a lower extent, TNF-α, causes cell death and that moxonidine protects against starvation- as well as IL-1β –induced mortality, mainly by maintaining membrane integrity, and in part, by improving mitochondrial activity. The protection involves activation of Akt, ERK1/2, p38, JNK, and iNOS. In contrast, moxonidine stimulates basal and IL-1β-induced fibroblast mortality by mechanisms that include inhibition of JNK and iNOS. Thus, apart from their actions on the central nervous system, imidazoline $I_1$-receptors are directly involved in cardiac cell growth and death, and may play an important role in cardiovascular diseases associated with inflammation.

**Keywords:** Cardiomyocytes; fibroblasts; moxonidine; imidazoline $I_1$- receptors; cytokines; apoptosis.

**Chemical compounds used in this article**

Moxonidine (PubChem CID: 4810)

**Abbreviations**

AGN192403 (2-endo-amino-3-exo-isopropylbicyclo[2.2.1]heptane)

ANP, atrial natriuretic peptide

DMEM, Dulbecco's Modified Eagle Medium

ERK1/2, extracellular signal-regulated protein kinase

FBS, Fetal Bovine Serum

IL-1β, Interleukin 1 beta

Imidazoline $I_1$-receptors, Imidazoline receptors type 1

iNOS, inducible nitric oxide synthase

JNK, c-Jun NH2-terminal protein kinase

LVH, left ventricular hypertrophy

MAPK, mitogen activated protein kinase

NO, nitric oxide

PI3K, phosphatidylinositol 3-kinase

RVLM, rostral ventrolateral medulla

S1P, sphingosine 1 phosphate

TNF-α, tumor necrosis factor alpha

## 1. Introduction

Left ventricular hypertrophy (LVH) and remodelling in hypertension and heart failure have been associated with increased circulating and cardiac expression of pro-inflammatory cytokines, tumor necrosis alpha (TNF-α) and interleukin (IL)-1β (Duerrschmid et al., 2013; Gullestad et al., 2012). These cytokines lead to cardiomyocyte hypertrophy and apoptosis in vivo and in vitro (Haudek et al., 2007; Hiraoka et al., 2001; Hu et al., 2009), and stimulation of cardiac fibroblast proliferation and differentiation into activated myofibroblasts, which produce large amounts of collagens. The ensuing structural changes, LVH and fibrosis, impede cardiac contractility and compromise cardiac performance. The intracellular signalling pathways include generation of reactive oxygen species (Khurana et al., 2014; Kohler et al., 2014), activation of phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B, PKB) (Chu et al., 2012; Tullio et al., 2013) and mitogen activated protein kinases (MAPKs): extracellular signal-regulated protein kinase (ERK), p38, and c-Jun $NH_2$-terminal protein kinase (JNK) (Chae et al., 2006; Haudek et al., 2007; Hiraoka et al., 2001; Sugden and Clerk, 1998; Yndestad et al., 2010).

Moxonidine is a centrally-acting sympatholytic imidazoline compound that shows higher affinity to non-adrenergic imidazoline $I_1$-receptors than $\alpha_2$-adrenergic receptors. Moxonidine is used in hypertension treatment, including hypertension complicated with LVH (reviewed in (Mukaddam-Daher, 2012). Our studies have shown that treatment of spontaneously hypertensive rats (SHR) with moxonidine improves cardiac performance, attenuates LVH and collagen accumulation, in association with a transient apoptotic effect in SHR ventricles. The effects also include a substantial reduction in left ventricular IL-1β and circulating TNF-α and IL-6 levels, as well as attenuated ventricular Akt and p38 phosphorylation and inducible nitric oxide synthase (iNOS) expression (Aceros et al., 2011; Mukaddam-Daher et al., 2009; Paquette et al., 2008). On the other hand, moxonidine improves cardiac performance in cardiomyopathic hamsters in association with differential inflammatory/anti-inflammatory responses that culminate in reduced cardiac apoptosis (Stabile et al., 2011). The cardioprotective effects of in vivo moxonidine may be secondary to inhibition of sympathetic nerve activity, reduced noradrenaline release and cytokine levels, and subsequently, their downstream actions. Moxonidine may also directly act on cardiac cells to modulate the actions

of cytokines. This hypothesis is supported by data showing that cardiac cells express cytokine receptors and imidazoline $I_1$-receptors (also known as nischarin), and in vitro moxonidine attenuates noradrenaline-induced cardiomyocyte apoptosis and fibroblast proliferation (Aceros et al., 2011). Accordingly, we sought to examine in primary cultures of neonatal rat cardiomyocytes and fibroblasts: i) the direct effect of moxonidine on cardiac cell viability and mechanisms involved, including the expression of imidazoline $I_1$-receptor/nischarin; and ii) to identify at what level moxonidine may regulate cytokine-induced cellular effects, focussing on their common signalling pathways, MAPKs, iNOS, and Akt (Aceros et al., 2011; Akira and Takeda, 2004; Edwards et al., 2001; Peng et al., 2009; Tesfai et al., 2011). The results demonstrate for the first time, the direct effect of moxonidine on cardiac cell viability. The mechanisms involve $I_1$-receptor activation and subsequent ERK and Akt activation in cardiomyocytes and ERK, p38, JNK, and Akt in fibroblasts. Furthermore, moxonidine modulates cytokine-induced effects on cardiomyocyte and fibroblast viability, through differential effects on cytokine-induced p38, JNK and iNOS.

## 2. Materials and methods

### *2.1. Cell Cultures*

Ventricular cardiomyocytes and fibroblasts were isolated, enzymatically digested, and purified from 1 to 2 day old Sprague Dawley rat pups, using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp, Lakewood, NJ, USA) following manufacturer instructions, as we previously described (Aceros et al., 2011). Recovered cells were pre-plated on plastic flasks twice, for 30 min each, to reduce non-myocyte cell numbers. The adherent cells are mostly fibroblasts while cardiomyocytes are in the suspension.

Isolated Cardiomyocytes seeded at a density of $2.5 \times 10^5$ per ml were cultured in Dulbecco's Modified Eagle Medium (DMEM), low glucose, 4 mM L-Glutamine, 1 mM Sodium Pyruvate (Invitrogen, Burlington, ON), supplemented with 10% FBS (Fetal Bovine Serum, Qualified, Canada Origin, Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (P/S) (Invitrogen, Burlington, ON, Canada). The medium was changed every 2 days.

Cytosine arabinoside (cyt Ara, 10 μM) was added throughout the cardiomyocyte culture period to inhibit non-cardiomyocyte outgrowth (Condorelli et al., 2002). Because of its possible toxicity (Gonzalez-Juanatey et al., 2004), and to rule out possible influence on cytokine-induced cell death, cyt Ara was omitted in some cultures. However, cytotoxicity assays, performed simultaneously did not show differences between the cells cultured with or without cyt Ara, at the concentration used, so results were pooled. Furthermore, microscopic evaluation of α-sarcomeric actin (mouse, Sigma-Aldrich A2172, Oakville, ON, Canada) confirmed purity (>95%) of cardiomyocyte cultures.

Fibroblasts reaching 70% confluence after 3 days of culture were sub-cultured to reduce the possible contamination by cardiomyocytes and endothelial cells, then seeded at a density of $1.25 \times 10^5$ per ml in six-well plates, in triplicate.

The cultured cardiomyocytes and fibroblasts were a pooled cell population from 11-15 neonatal rat ventricles in each experiment. Four to five independent experiments done in triplicate were performed for each condition. All experimental procedures were approved by the Ethical Committee of CRCHUM following the Canadian Guidelines.

*2.2. PCR Analysis*

Nischarin mRNA (accession number NM_022656.2) was detected in cardiomyocytes collected after 3 days of isolation and in fibroblasts on first and second passages. Normal C57BL/6 mouse (Charles-river, St-Constant, QC) brain and heart were used as positive control for nischarin mRNA.

Cardiomyocytes, seeded at a density of $2.5 \times 10^5$ per ml and fibroblasts at a density of $1.25 \times 10^5$ per ml in six-well plates, in triplicate, as well as tissues were collected and snap frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent; equal amounts of RNA were reverse transcribed to cDNA using Maloney murine leukemia virus reverse transcriptase (M-MLV reverse transcriptase), then amplified with Taq polymerase (all from Invitrogen, Burlington, ON, Canada). cDNA was then amplified by PCR using the following primers for nischarin:

Fw: AGAGGCTCCAGCTCCAAAC; Rv: TTCTTGTCCCCAAATTCTGA, with an expected product of 706 bp. After 40 cycles with an annealing temperature of 60 °C, the

product was migrated using a 1% agarose gel with SYBR safe DNA gel stain (Invitrogen, Burlington, ON, Canada). S18 amplification was used as loading control. Images were taken with Typhoon trio imager (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada).

*2.3. Cell treatment*

Cardiomyocytes on day 3 after isolation and fibroblasts at the second passage were treated and harvested simultaneously under identical experimental conditions, using the same concentrations and duration of treatments. All cells were grown in DMEM containing 10% FBS, then rendered quiescent by incubation overnight in DMEM supplemented with 0.1% FBS. All procedures were performed using DMEM+0.1%FBS, hereafter termed DMEM, as control vehicle, unless otherwise specified.

Synchronized quiescent cells were then incubated for different time points, either in freshly prepared DMEM alone or containing $10^{-7}$ or $10^{-5}$ M moxonidine (Solvay Pharmaceuticals, Hannover, Germany), TNF-α (10 ng/ml; Recombinant rat, catalog number CLR08, Cedarlane Canada, Burlington, ON, Canada), or IL-1β (5 ng/ml; Recombinant rat, catalog number EN-RR202420, Thermo Scientific, Rockford, IL, USA), each alone or in combination with moxonidine. The concentrations of TNF-α and IL-1β and incubation times were chosen following published reports showing increased reactive oxygen species production and iNOS expression in neonatal rat cardiomyocytes, respectively (Higuchi et al., 2002), (LaPointe and Sitkins, 1996). Incubation in DMEM supplemented with 10%FBS (DMEM+10%FBS, replete condition) or $H_2O_2$ ($10^{-4}$ M), which induces neonatal cardiomyocyte apoptosis (Long et al., 2004), were used as positive and negative controls. Incubation with lipopolysaccharides from Escherichia coli 0111:B4 (LPS, 100 ng/ml) (Sigma-Aldrich, Oakville, ON, Canada) was used as positive control for iNOS expression (Li et al., 2009b). The participation of imidazoline $I_1$-receptor/nischarin was confirmed by incubating the cells in the presence of AGN192403 (2-endo-amino-3-exo-isopropylbicyclo[2.2.1]heptane, 10 μM, Tocris Bioscience, Ellisville, MO, USA), an imidazoline $I_1$-receptor antagonist (Aceros et al., 2011).

*2.4. Western blot analysis*

To investigate the signalling pathways activated by moxonidine and interaction with cytokines, cardiomyocytes and fibroblasts were treated for 15 min or 24h and 48h, with indicated products freshly prepared in DMEM. The cells were harvested, rinsed then lysed with a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM EDTA (Ethylenediamine tetraacetic acid) and a protease inhibitor cocktail.

Equal amounts of proteins (20 µg for MAPKs and Akt, 50 µg for iNOS and nischarin) separated on 10% SDS-polyacrylamide gel were transferred to nitrocellulose membrane, then blocked in 5% non-fat milk, for 6 h, at 4°C. After washing, the membrane was probed overnight at 4°C with antibodies recognizing the following antigens: Rabbit polyclonal Bax antibody (20 kDa, 1:1000); rabbit polyclonal phospho Akt-1 at Ser473 (60 kDa, 1:1000), mouse monoclonal phospho-p44/42 mitogen-activated protein kinase extracellular signal-regulated kinase (ERK1/2) (Thr202/Tyr204, 44 and 42 kDa, 1:1000), rabbit polyclonal phospho p38 mitogen-activated protein kinase (p38 MAPK) (Thr180/Tyr182, 38 kDa, 1:1000), rabbit polyclonal phospho c-Jun N-terminal kinase (JNK) (Thr183-Tyr185, 54 and 46 kDa, 1:1000) (all from cell signalling, Danvers, MA, USA), purified mouse anti Bcl2 (26 kDa, 1:1000) (BD transduction Laboratories, Mississauga, ON, Canada) and rabbit polyclonal NOS2 inducible nitric oxide synthase (iNOS, 130 kDa, 1:1000; Santa Cruz, Dallas, TX, USA). Imidazoline $I_1$-receptor protein expression was identified using a monoclonal mouse antibody raised against the murine form, nischarin (250 kDa, 1:1000; BD Pharmigen, Mississauga, ON, Canada). Antibodies against total non-phosphorylated proteins performed in preliminary experiments were found not to be altered by 15 min incubation, therefore omitted in further Westerns. Loading was assessed by GAPDH (rabbit anti-GAPDH, 36 kDa 1:10,000; Sigma-Aldrich, Oakville, ON, Canada) or β-actin (mouse Anti-actin, 42 kDa, 1:10,000; Chemicon, Billerica, MA, USA). Appropriate peroxidase-conjugated secondary antibodies were used for detection, and visualized by ECL-Plus or ECL-Advanced chemiluminescence detection (GE Amersham, Baie d'Urfe, QC, Canada). The density of the bands was quantified and normalized to corresponding DMEM-incubated control cells (considered as 100%).

*2.5. Analysis of Cell Viability*

Cell viability was examined in 3 methods that detect different parameters of cellular toxicity/viability. Flow cytometry with fluorescein isothiocyanate-(FITC)-conjugated annexin V and propidium iodide stains detect the rate of apoptosis and necrosis, respectively. Colorimetric MTT assay detects cell membrane integrity and enzyme activity in mitochondria, reflecting the degree of change in mitochondrial function, which also correlates with cell division/proliferation. The Live/Dead assay discriminates live cells from dead ones on the basis of membrane integrity, using calcein acetoxymethyl ester (Calcein AM) stain to detect intracellular esterase activity, and ethidium homodimer-1 (EthD-1) stain to detect cell membrane integrity.

Cells were incubated for 48 h in DMEM vehicle, moxonidine, and cytokines, each alone or in combination. In order to determine which of the signaling pathways was important for cell growth and death, the Live/Dead assay also included pre-treatment for 1h with antagonists/inhibitors. AGN192403 (10 μM, Tocris Bioscience, Ellisville, MO, USA); PD98059 (2-(2'-Amino-3'-methoxy)-flavone) (10 μM), a selective inhibitor of MEK1/MEK2, the upstream activator of ERK1/ERK2; SB203580 (4-(4'-fluorophenyl)-2-(4'methylsulfinylphenyl))-5-(4'-pyridyl)imidazole) (10 μM), an inhibitor of p38-MAPK; SP600125 (Anthra(1,9-cd)pyrazol-6(2H)-one) (10 μM), a JNK inhibitor; wortmannin (0.5 μM), a PI3K inhibitor, all from Cell signalling (Danvers, MA, USA), and S-Methylisothiourea Sulfate (SMT, 1 μM, Calbiochem, Darmstadt, Germany), an iNOS inhibitor. The concentrations of the products used in this study are consistent with the range previously used on cardiac myocytes *in vitro* (Engelbrecht et al., 2004).

*2.5.1 Flow cytometry*

The rate of apoptosis was quantitatively evaluated by flow cytometry using the FITC AnnexinV Apoptosis Detection Kit I (BD Biosciences, Mississauga, ON, Canada), following manufacturer instructions, as we previously described (Aceros et al., 2011). After 48 h of incubation with the products, cells were washed with PBS, trypsinized then centrifuged. The pellet was incubated with 5 μl propidium iodide (PI, 50 μg/ml) and 5 μl annexin V, in 100 μl Annexin binding buffer, for 15 min in the dark. Cells were next analyzed for

annexin/propidium iodide binding by flow cytometry using a BD LSRII with the software FACS DIVA version 5.0.2 (BD, Mississauga, ON). Annexin binding represents plasma membrane phosphatidylserine externalization to the outer leaflet of the plasma membrane, an early step in the apoptotic process, whereas propidium iodide, a fluorescent nucleic acid binding dye, excluded from live cells, stains cells in the late stages of apoptosis and necrosis (DeBiasi et al., 2004). Cells in each category were expressed as percentages of the total number of stained cells. The values were then expressed as percentages of DMEM-incubated control.

*2.5.2 MTT assay*

After 48 h treatment, viable cells were detected by incubation with 10 µl tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT), in phenol red-free culture medium, for 4h, at 37°C (MTT Cell Proliferation Assay, ATCC, Manassas, VA, USA). Metabolically oxidized purple-colored formazan crystal, which forms only in the presence of intact cell membranes and functional mitochondria, were solubilized in 100 µl of 10% SDS/0.01 M HCl and read from an absorbance plate reader (Biotek Synergy HT with the software Gen5 data analysis, Winooski, VT, USA) at a wavelength of 570 nm, within 24h.

*2.5.3 LIVE/DEAD assay*

After 48h incubation, 0.5 mM calcein acetoxymethyl ester (Calcein AM) and 4 mM and ethidium homodimer-1 (EthD-1) (LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells, Invitrogen, Burlington, ON, Canada) were added to the culture dishes for 30 min, and incubated at 37°C, then the cells were immediately viewed with a fluorescent inverted microscope, Olympus IX 71 (Center Valley, PA, USA). The non-fluorescent calcein-AM is converted into green fluorescent polyanionic calcein by intracellular esterase, indicating active cell metabolism. Ethidium homodimer is excluded by viable cells but permeates damaged cell membranes, binds to nucleic acids, and results in red fluorescence. The number of green and red colored cells was counted in three wells per treatment and 4 fields per well, at 100-fold magnification.

*2.6. Statistical analysis*

All results are presented as mean±S.E.M. Statistical comparisons among multiple groups were performed using analysis of variance (ANOVA) followed by Newman–Keuls test for multiple comparisons. The criterion of statistical significance was $P<0.05$. All analyses were carried out using Prism 4.0 (GraphPad Software, San Diego, CA, USA).

## 3. Results

*3.1. Expression of nischarin in neonatal cardiomyocytes and fibroblasts*

Nischarin mRNA expression was detected in mouse brain and heart at 706 bp, confirming previous results (Alahari et al., 2000). Nischarin mRNA was also detected in neonatal rat cardiomyocytes and second passage fibroblasts. Nischarin mRNA was reduced after 24 h incubation with moxonidine in cardiomyocytes, but not altered in fibroblasts (Fig. 1). Imidazoline $I_1$-receptor/nischarin expression and regulation was further explored by Western blot. Fig. 2A shows that nischarin protein expression is reduced in cardiomyocytes and increased in fibroblasts after 24 h incubation with moxonidine.

Furthermore, cardiomyocyte incubation with noradrenaline ($10^{-5}$ M) induced nischarin protein over-expression (134±9%, $P<0.01$ vs. DMEM) and the effect was reversed by $10^{-5}$ M moxonidine (58±15%, $P<0.01$ vs. noradrenaline). However, TNF-α and IL-1β, each alone or in combination with moxonidine did not modify nischarin expression in cardiomyocytes. On the other hand, nischarin expression in fibroblasts was not affected by noradrenaline but significantly reduced by combined noradrenaline and moxonidine (31±7%; $P<0.01$ vs. both, DMEM and noradrenaline). Nischarin expression was reduced by TNF-α, an effect reversed by moxonidine. However, nischarin expression in fibroblasts was not affected by IL-1β alone or in combination with moxonidine (Fig. 2B). These results demonstrate differential regulation of nischarin by hypertension- and heart failure-associated stimuli.

*3.2. Effects of moxonidine on signalling proteins in cardiomyocytes and fibroblasts*

MAPK and the PI3K/Akt pathways play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and various stresses in

cardiomyocytes and fibroblasts (Clerk et al., 1999; Thaik et al., 1995). ERK1/2 and JNK as well as Akt are implicated in imidazoline $I_1$-receptor signalling in neuronal pheochromocytoma PC12 cell line (Edwards et al., 2001; Tesfai et al., 2011). But imidazoline $I_1$-receptor signalling in different cells may lead to the activation of distinct downstream targets resulting in different physiological effects and the relative contribution of each pathway may vary in different cell types. Therefore, we investigated whether similar imidazoline $I_1$-receptor signalling pathways occur in cardiomyocytes and fibroblasts

Western blot analysis revealed that 15 min incubation of cardiomyocytes with $10^{-5}$ but not $10^{-7}$ M moxonidine causes an increase in Akt and ERK1/2 but not JNK or p38 phosphorylation. ERK1/2 activation is inhibited by imidazoline $I_1$-receptor antagonist, implicating imidazoline $I_1$-receptors in this effect (Fig. 3). Because moxonidine at $10^{-5}$ but not $10^{-7}$ M concentration activated cellular pathways in cardiomyocytes, this concentration was used in further experiments.

Akt and ERK1/2 are important regulators of cell viability. Akt provides resistance against oxidative stress as well as apoptosis in neonatal rat cardiomyocytes, by modifying the balance of apoptotic/anti-apoptotic mitochondrial enzymes Bax and Bcl2. Therefore, we measured the protein expression of these enzymes. Western blot analysis revealed that $10^{-5}$ M moxonidine, for 24 h, inhibits apoptotic protein Bax (62±6%; n=6-9; $P<0.01$) and increases anti-apoptotic Bcl2 (150±15%; n=6-9; $P<0.03$), thus reducing Bax to Bcl2 ratio, consistent with an anti-apoptotic effect of moxonidine in cardiomyocytes (Fig. 4).

Incubation of fibroblasts with $10^{-5}$ M moxonidine for 15 min results in ~ 2 fold increase in ERK1/2, p38, JNK, and Akt phosphorylation. In contrast to cardiomyocytes, 24h incubation of fibroblasts with moxonidine alone increases Bax (189±12%, n=9; $P<0.01$) and attenuates Bcl2 (86±4%; n=9), resulting in a higher Bax to Bcl2 ratio. These results are consistent with apoptotic effects of moxonidine in fibroblasts (Fig. 4).

*3.3. Effects of moxonidine and cytokines on signalling proteins in cardiomyocytes and fibroblasts*

The interaction of moxonidine and cytokines was investigated in cardiomyocytes and fibroblasts. Fig. 5 shows that 15 min cardiomyocyte incubation with IL-1β, but not TNF-α,

causes an increase in phosphorylated Akt and MAPKs. Incubation with moxonidine potentiates IL-1β-induced activation of p38 and JNK while it activates Akt and ERK1/2 in the presence of TNF-α. These differential effects indicate stimulus-dependent imidazoline $I_1$-receptor signalling in cardiomyocytes.

Fibroblast incubation with IL-1β activates MAPKs and Akt, but TNF activates MAPKs. Moxonidine opposes IL-1β-induced Akt and JNK and TNF-α-induced ERK1/2 and JNK, but potentiates TNF-α-induced Akt and p38 phosphorylation (Fig. 6).

*3.4. Effects of moxonidine and cytokines on iNOS expression*

MAPKs may activate the constitutive NOS isoforms, eNOS and nNOS (Kan et al., 2008). However, cytokine-induced MAPK and Akt activation in cardiomyocytes and fibroblasts enhances iNOS production (Gustafsson and Brunton, 2000; Kan et al., 1999; Lizano et al., 2013; Shindo et al., 1995). Accordingly, we investigated whether moxonidine/imidazoline $I_1$-receptor activation modulates the cytokine-iNOS pathway. The iNOS protein expression was measured in cells incubated with moxonidine alone or in combination with IL-1β or TNF-α, for 24 and 48 h. Cells incubated in 100 mg/ml LPS were used as a positive control. Western blot revealed that iNOS protein expression was detected in DMEM-incubated cardiomyocytes and significantly increased by LPS (~750%). The iNOS expression in cardiomyocytes was not affected by 24h incubation with $10^{-7}$ and $10^{-5}$ M moxonidine, however it was increased by $10^{-5}$ M moxonidine by 48h (247±50%, $P<0.02$) (Fig. 7).

Incubation with IL-1β and TNF-α for 24 h significantly increased iNOS expression in cardiomyocytes. IL-1β- but not TNF-α-induced iNOS was potentiated by $10^{-5}$ M moxonidine (Fig. 7).

Compared to DMEM-incubated fibroblasts (considered as 100%), moxonidine reduced iNOS expression to 75±5% and 9±3% ($P<0.01$) by 24 and 48h, respectively. Incubation with cytokines for 24 h increased iNOS to much higher levels than those measured in cardiomyocytes. Co-incubation with moxonidine did not affect TNF-α-induced iNOS, but, inhibited IL-1β-induced iNOS (Fig. 7).

The differential effects of moxonidine alone and on IL-1β- and TNF-α-induced signalling proteins in fibroblasts, in a manner different from those observed in cardiomyocytes indicate that moxonidine/imidazoline $I_1$-receptor signalling is stimulus-specific as well as cell-type specific.

*3.5. Effect of moxonidine and cytokines on cardiomyocyte viability*

The signalling proteins altered by moxonidine and cytokines are implicated in cell growth and death. Accordingly, we measured the influence of moxonidine on cell viability and interaction with cytokines. Incubation in DMEM+10%FBS (serum-replete) indicated normal growth and $10^{-4}$ M $H_2O_2$ in starvation conditions (DMEM) indicated cell death.

Compared to 48 h serum-starved cardiomyocytes, incubation in serum-replete conditions resulted in 23±2% lower annexin positive/PI negative cells (early apoptosis) and 45±5% in PI positive binding (necrosis). Cardiomyocyte viability/mitochondrial function was mildly, yet significantly higher (12±2%, $P<0.01$) in serum replete myocytes. The LIVE/DEAD assay revealed higher metabolic activity and 50% lower cellular and nuclear membrane damage. Similar trends were obtained with fibroblasts. These results show that starvation conditions result in cardiomyocyte and fibroblast death by inducing apoptosis and necrosis, secondary to membrane damage, and in part, to reduced mitochondrial function and cell metabolic activity. These effects are abrogated in the presence of serum, indicating that the cells are responsive to growth conditions.

In serum starvation conditions, 48h incubation of cardiomyocytes with $10^{-7}$ and $10^{-5}$ M moxonidine resulted in lower percentage of cells in early apoptosis and mildly higher percentage in necrosis, culminating in a mild increase in percent alive cells. Incubation with AGN192403 prevented these actions, indicating imidazoline $I_1$-receptor mediated actions (Fig. 8). As expected, cardiomyocyte incubation with $H_2O_2$ increased the rate of early apoptosis and to a lower extent cell necrosis. Both actions were prevented by co-incubation with $10^{-5}$ M moxonidine (Fig. 8). Although $H_2O_2$ was used as a cell death control, this finding is consistent with an anti-oxidant effect of moxonidine in cardiomyocytes.

The MTT assay revealed that moxonidine had no effect, but IL-1β and TNF-α mildly reduced (~15%) cardiomyocyte viability/mitochondrial activity, effects prevented by co-incubation with $10^{-5}$ M moxonidine (Fig. 9).

Fig. 10A shows fluorescent micrographs of calcein and ethidium homodimer staining in cardiomyocytes, reflecting intracellular esterase activity in live cells as well as cell death due to compromised membrane integrity, respectively. Calcein staining was not affected by either treatment. Percent red stained cell number, was not affected by moxonidine, however it was significantly increased upon co-incubation of moxonidine with AGN192403, SP600125, and SMT, and opposed the toxic effect of SB203580, indicating that moxonidine protects cardiomyocyte membrane integrity through imidazoline $I_1$-receptors and activated p38, JNK, and iNOS (Fig. 10B).

Incubation with IL-1β caused 70% increase in the number of ethidium stained cells, reflecting damaged cell membranes (Fig. 11A). IL-1β-induced cardiomyocyte death was prevented by SB203580 and SMT, implicating p38 and iNOS in this action, effects consistent with previous reports (Arstall et al., 1999; Shindo et al., 1995). Moxonidine attenuated IL-1β-induced cell death, an effect prevented by wortmannin, PD98059, SB203580, and SMT. Therefore, moxonidine protects cardiomyocytes against IL-1β-induced membrane damage through Akt, ERK1/2, p38, and iNOS.

Similarly, TNF-α caused an increase in cell death, but of a smaller magnitude (~20%) and the effect was prevented by co-incubation with moxonidine (Fig. 11B). The ERK1/2 inhibitor, PD98059 opposed moxonidine's effect, implicating ERK1/2 in the protective effect of moxonidine against TNF-α-induced cell death. Noteworthy, among the antagonists used SB203580, at the same concentration which did not affect metabolic activity, potentiated starvation-induced membrane damage, opposed membrane damage induced by IL-1β as well as protection by moxonidine.

*3.6. Effect of moxonidine and cytokines on fibroblast viability*

Incubation with $10^{-5}$ M moxonidine 48 h in serum replete conditions did not affect fibroblast apoptosis determined by annexin staining and flow cytometry, however, necrosis was lowered (~20%). In contrast, in starvation conditions, $10^{-5}$ M moxonidine caused an

increase in PI positive cells (145%±7, P<0.02) after 24h and did not change further by 48h (data not shown).

Moxonidine, IL-1β, and TNF-α, each alone, mildly, yet significantly, reduced (~15%, P<0.01) fibroblast viability/mitochondrial activity/proliferation measured by MTT. Co-incubation of cytokines with moxonidine did not result in further change (data not shown). However, calcein staining (Fig. 12A) revealed that fibroblast cellular activity was induced by moxonidine but inhibited by cytokines. Moxonidine did not alter IL-1β-reduced cellular activity but opposed that of TNF-α. Fig. 12B shows that ethidium homodimer stained cell number was more detected in fibroblasts incubated with moxonidine (143±20%; P<0.05) and to a greater extent in fibroblasts incubated with IL-1β and TNF-α than in DMEM control (100%). Moxonidine potentiated the action of IL-1β, but opposed that of TNF-α.

The signalling protein inhibitors, PD98059, SB203580, and wortmannin at concentrations which did not affect metabolic activity or membrane integrity in cardiomyocytes, were very toxic to fibroblasts, rendering unexplainable results. SP600125 did not affect basal and SMT reduced cell death, and both potentiated cell death induced by moxonidine alone and in combination with IL-1β and TNF-α. These results suggest that in the presence of cytokines, fibroblast death by moxonidine involves inhibition of JNK and iNOS. Further studies using lower concentrations of the inhibitors are required to evaluate the mechanisms that mediate imidazoline and cytokine actions in fibroblasts. However, these results reveal that fibroblasts are more sensitive to these inhibitors than cardiomyocytes.

## 4. Discussion

Our results provide evidence that moxonidine protects cultured neonatal cardiomyocytes against serum starvation-induced apoptosis as well as IL-1β- and TNF-α-reduced viability, mainly by maintaining membrane integrity, and in part, by improving mitochondrial activity. The protection primarily involves PI3K/Akt, ERK1/2, p38, and iNOS, in IL-1β-incubated cells, but activation of ERK1/2 in TNF-α-incubated cells. In contrast, moxonidine stimulates fibroblast mortality by mechanisms that include JNK and iNOS, and potentiates IL-1β- but opposes TNF-α-induced fibroblast mortality. Because the actions of

moxonidine are selectively mediated by imidazoline $I_1$-receptors, these studies point to an interaction between cytokines and cardiac imidazoline $I_1$-receptors.

We have previously identified functional imidazoline $I_1$-receptors in the heart (El-Ayoubi et al., 2002; Mukaddam-Daher et al., 1997; Mukaddam-Daher et al., 2006) and in primary cardiac cells (Aceros et al., 2011). The present study extends to assess cardiac cell imidazoline $I_1$-receptor/nischarin expression, regulation, and signalling. Herein, we detect nischarin mRNA expression in the heart, both in cardiomyocytes and fibroblasts and regulation by moxonidine. We also show that nischarin protein expression is reduced by moxonidine in cardiomyocytes but increased in fibroblasts. Also, nischarin levels in cardiomyocytes are increased by noradrenaline but not cytokines, while nischarin levels in fibroblasts are reduced by TNF-α and not affected by noradrenaline and IL-1β. These results demonstrate that imidazoline $I_1$-receptor/nischarin regulation is cell- and stimulus-specific. They are also consistent with *in vivo* studies revealing imidazoline $I_1$- receptor/nischarin up-regulation in the heart of genetically hypertensive and heart failure animals and differential modification by moxonidine (El-Ayoubi et al., 2002; Stabile et al., 2011).

Imidazoline $I_1$-receptor/nischarin regulation has been previously investigated in other cell types. Piletz et al. (2008) reported that imidazoline $I_1$-receptors are increased in platelets and frontal cortex of depressed patients and this up-regulation is normalized after antidepressant drug treatments. Altered imidazoline $I_1$-receptor expression reflects mood disorders (Piletz et al., 2008). Also, Alahari's group found repressed nischarin expression in a breast cancer cell line (BT-474) that was increased in a dose-dependent manner upon inhibition of PI3K/Akt by LY294002 (Jin et al., 2013). Accordingly, we may speculate activation of Akt by moxonidine, at least in cardiomyocytes, suppresses nischarin levels. However, the exact mechanisms involved in the observed changes in nischarin expression remain to be investigated.

Investigation of moxonidine signalling pathways in cardiomyocytes revealed that 15 min incubation with moxonidine causes an increase in ERK1/2 and Akt but not JNK or p38 phosphorylation. On the other hand, moxonidine activates Akt, ERK1/2, JNK, and p38 in fibroblasts. Long term incubation with moxonidine also increases iNOS protein expression in cardiomyocytes while it decreases it in fibroblasts. Importantly, whereas moxonidine and

imidazoline $I_1$-receptor signalling through ERK, JNK, and Akt has been shown in PC12 cell line (Edwards et al., 2001; Tesfai et al., 2011), to our knowledge, this is the first study that investigates these proteins as well as p38 and iNOS expression and regulation by moxonidine in primary cardiac cells.

Our results show that moxonidine differentially interacts with cytokines and in a cell-specific manner. Further studies are necessary to pinpoint the sequence of actions of moxonidine and the signalling pathways involved downstream of imidazoline $I_1$-receptor in inflammatory conditions. However, the interaction of moxonidine and cytokines may occur at any one of the common mediators. Imidazoline $I_1$-receptors as well as IL-1β and TNF-α receptors, are coupled to PC-PLC signalling pathways (Cobb et al., 1996; Ernsberger, 1999; Zhang et al., 2001); signalling in both involves sphingomyelin-ceramide pathway (Krown et al., 1996; Molderings et al., 2007), and both stimulate the increase in MAPKs and PI3K activity and downstream Akt, the transcription factor nuclear factor -κB (NF-κB), and iNOS (Edwards et al., 2001; Gustafsson and Brunton, 2000; Kan et al., 1999; Peng et al., 2009; Shindo et al., 1995; Tesfai et al., 2011).

The present studies also investigated the interaction between cytokines and moxonidine as well as downstream signalling proteins on cardiac cell viability. Cytokines have been shown to increase, decrease, or not change cardiomyocyte survival (Higuchi et al., 2002; Ing et al., 1999; Krown et al., 1996). These heterogeneous results depend on the experimental conditions, such as the use of neonatal or adult rat cardiomyocytes, presence or absence of serum, cytokine concentration, exposure time, culture plate coating, as well as by the complex intracellular mechanisms which mediate cytokine actions. TNF-α and IL-1β primarily bind to respective receptors to modulate the activities of common intracellular signalling pathways involved in cell apoptosis or anti-apoptosis. These pathways include cytokine activation of ERK1/2, which mediates cell growth and survival signals, and p38 and JNK, which are generally pro-apoptotic (Bogoyevitch et al., 1996; Dhingra et al., 2007; Sugden and Clerk, 1998; Yndestad et al., 2010). Cytokines activate the transcription factor NF-κB, which in turn, is apoptotic and anti-apoptotic. NF-κB induces iNOS expression and NO production, which contributes to cell death or blocks the apoptosis signalling cascade (Haudek et al., 2007; Hiraoka et al., 2001; Hu et al., 2009; Lu et al., 2008). In addition, cytokines increase the

intracellular production of the second messenger sphingolipid metabolites: ceramide, sphingosine, and the phosphorylated derivative of sphingosine, S1P (Krown et al., 1996). Ceramide and sphingosine induce growth arrest and apoptosis (Hannun and Obeid, 2008), while S1P inhibits apoptosis and promotes proliferation (Spiegel and Milstien, 2003). Thus, a dynamic balance between pro-apoptotic and anti-apoptotic pathways determines the fate of the cells whether to die or survive.

In the conditions employed, IL-1β, and to a lower extent, TNF-α, reduced cardiomyocyte viability and caused cell death, mainly by causing cell membrane damage. Cardiomyocyte mortality induced by IL-1β was opposed by the specific inhibitors SP600125, SB203580, and SMT, confirming the prominent participation of JNK, p38, and iNOS in IL-1β-induced cell death in several cell types, including cardiomyocytes (Ing et al., 1999; Luss et al., 1995; Shindo et al., 1995). IL-1β-induced cardiomyocytes death was also opposed by moxonidine. However, to our surprise, cell death was higher upon inhibition of JNK, p38, and iNOS in cardiomyocytes incubated with moxonidine alone and much more in cells incubated with moxonidine in the presence of IL-1β. Accordingly, moxonidine's potentiation of IL-1β-induced JNK, p38, and iNOS in cardiomyocytes observed by Western blot may be compensatory cytoprotective mechanisms, since their inhibition potentiates cell death.

In fact, the implication of p38, JNK, and iNOS in mediating cardiomyocyte survival has been previously shown (Jones and Bolli, 2006; Li et al., 2009a; Markou et al., 2011; Munoz et al., 2010; Wei et al., 2011). In ischemia/reperfusion conditions, blocking p38 activity in adult rat cardiomyocytes reverses the protection induced by phosphodiesterase inhibition (Markou et al., 2011); and JNK activity can be both, protective or deleterious, depending on the availability of glucose and ATP (Wei et al., 2011). Studies of ischemic preconditioning and *iNOS* gene transfer have clearly shown that iNOS protects against myocardial infarction and that the beneficial effect of several agents, including statins and phosphodiesterase-5 inhibitors, on the heart are associated with induced iNOS activity, more significantly linked to late preconditioning (Jones and Bolli, 2006; Li et al., 2009a). Protection by iNOS may include reduction of reactive oxygen species generation or activation of NF-κB-mediated survival pathway (Li et al., 2009a). Again, the resulting cell survival or death depends on the balance between associated pro- and anti-survival proteins.

In this respect, based on published data and our present results, we propose that moxonidine protects against cytokine-induced cardiomyocyte death, as follows (Fig. 13): IL-1β alone increases p38, JNK, and iNOS and results in cell death. Moxonidine causes an increase in the anti-apoptotic protein Bcl2 and reduces the apoptotic protein Bax, favoring cardiomyocyte survival. Moxonidine also potentiates IL-1β-induced p38, JNK and iNOS. Once iNOS is expressed, it produces large quantities of nitric oxide (NO), which can be both pro- and anti-apoptotic, depending on quantities and downstream signalling pathways: NO combining with oxidants generates the free radicals, peroxynitrites (ONOO-), inhibits mitochondrial enzymes, and induces cell death. On the other hand, NO can be cytoprotective, inducing Bcl2, which normally protects cells from apoptotic triggers. Through activation of the pro-survival/anti-apoptotic signals, as well as through its antioxidant effect, moxonidine may shift the balance towards the anti-apoptotic effect of NO. Opposite mechanisms may take place in fibroblasts, where moxonidine increases Bax, reduces basal and IL-1β-induced JNK and iNOS and does not exert an anti-oxidant effect, thus the balance is shifted towards cell death. Further studies are required to confirm these postulated survival/death pathways which may be strongly re-enforced by measurement of the effect of moxonidine and cytokines on nitric oxide and reactive oxygen species production as well as Bax, Bcl2 and downstream caspase3 activity, the final common death pathway.

## 5. Conclusion

Studies on isolated neonatal cardiac cells in culture cannot be extrapolated to whole animals, especially in those with cardiac disease, where myriad of diverse mediators are activated. However, the finding that moxonidine/imidazoline $I_1$-receptor protects cardiomyocytes and favors elimination of myofibroblasts in inflammatory conditions, is consistent with regression of LVH and fibrosis and improved cardiac function in SHR (Aceros et al., 2011) and heart failure hamsters (Stabile et al., 2011). Moreover, the current observations implicate imidazoline $I_1$-receptors in cardiac cell growth and death independently of their role in blood pressure regulation. They also establish the foundation that cardiac imidazoline $I_1$-receptors differentially interact with cytokines, and suggest that therapy should take into consideration the nature of the inflammatory disorder and the cell type involved.

Further studies are required to confirm the beneficial effects of moxonidine in a wide range of animal models of inflammation and organ injury.

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## References


Aceros, H., Farah, G., Cobos-Puc, L., Stabile, A.M., Noiseux, N., Mukaddam-Daher, S., 2011. Moxonidine improves cardiac structure and performance in SHR through inhibition of cytokines, p38 MAPK and Akt. Br. J. Pharmacol. 164, 946-957.

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499-511.

Alahari, S.K., Lee, J.W., Juliano, R.L., 2000. Nischarin, a novel protein that interacts with the integrin alpha5 subunit and inhibits cell migration. J. Cell Biol. 151, 1141-1154.

Arstall, M.A., Sawyer, D.B., Fukazawa, R., Kelly, R.A., 1999. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ. Res. 85, 829-840.

Bogoyevitch, M.A., Gillespie-Brown, J., Ketterman, A.J., Fuller, S.J., Ben-Levy, R., Ashworth, A., Marshall, C.J., Sugden, P.H., 1996. Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. Circ. Res. 79, 162-173.

Chae, H.J., Ha, K.C., Kim, D.S., Cheung, G.S., Kwak, Y.G., Kim, H.M., Kim, Y.M., Pae, H.O., Chung, H.T., Chae, S.W., Kim, H.R., 2006. Catalase protects cardiomyocytes via its inhibition of nitric oxide synthesis. Nitric Oxide 14, 189-199.

Chu, M., Koshman, Y., Iyengar, R., Kim, T., Russell, B., Samarel, A.M., 2012. Contractile Activity Regulates Inducible Nitric Oxide Synthase Expression and NO(i) Production in Cardiomyocytes via a FAK-Dependent Signaling Pathway. J. Signal. Transduct. 2012, 473410.

Clerk, A., Harrison, J.G., Long, C.S., Sugden, P.H., 1999. Pro-inflammatory cytokines stimulate mitogen-activated protein kinase subfamilies, increase phosphorylation of c-Jun and ATF2 and upregulate c-Jun protein in neonatal rat ventricular myocytes. J. Mol. Cell. Cardiol. 31, 2087-2099.

Cobb, R.R., Felts, K.A., Parry, G.C., Mackman, N., 1996. D609, a phosphatidylcholine-specific phospholipase C inhibitor, blocks interleukin-1 beta-induced vascular cell adhesion molecule 1 gene expression in human endothelial cells. Mol. Pharmacol. 49, 998-1004.

Condorelli, G., Morisco, C., Latronico, M.V., Claudio, P.P., Dent, P., Tsichlis, P., Condorelli, G., Frati, G., Drusco, A., Croce, C.M., Napoli, C., 2002. TNF-alpha signal transduction in rat neonatal cardiac myocytes: definition of pathways generating from the TNF-alpha receptor. Faseb J. 16, 1732-1737.

DeBiasi, R.L., Robinson, B.A., Sherry, B., Bouchard, R., Brown, R.D., Rizeq, M., Long, C., Tyler, K.L., 2004. Caspase inhibition protects against reovirus-induced myocardial injury in vitro and in vivo. J. Virol. 78, 11040-11050.

Dhingra, S., Sharma, A.K., Singla, D.K., Singal, P.K., 2007. p38 and ERK1/2 MAPKs mediate the interplay of TNF-alpha and IL-10 in regulating oxidative stress and cardiac myocyte apoptosis. Am. J. Physiol. Heart Circ. Physiol. 293, H3524-3531.

Duerrschmid, C., Crawford, J.R., Reineke, E., Taffet, G.E., Trial, J., Entman, M.L., Haudek, S.B., 2013. TNF receptor 1 signaling is critically involved in mediating angiotensin-II-induced cardiac fibrosis. J. Mol. Cell. Cardiol. 57, 59-67.

Edwards, L., Fishman, D., Horowitz, P., Bourbon, N., Kester, M., Ernsberger, P., 2001. The I1-imidazoline receptor in PC12 pheochromocytoma cells activates protein kinases C, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK). J. Neurochem. 79, 931-940.

El-Ayoubi, R., Gutkowska, J., Regunathan, S., Mukaddam-Daher, S., 2002. Imidazoline receptors in the heart: characterization, distribution, and regulation. J. Cardiovasc. Pharmacol. 39, 875-883.

Engelbrecht, A.M., Niesler, C., Page, C., Lochner, A., 2004. p38 and JNK have distinct regulatory functions on the development of apoptosis during simulated ischaemia and reperfusion in neonatal cardiomyocytes. Basic Res. Cardiol. 99, 338-350.

Ernsberger, P., 1999. The I1-imidazoline receptor and its cellular signalling pathways. Ann. N. Y. Acad. Sci. 881, 35-53.

Gonzalez-Juanatey, J.R., Pineiro, R., Iglesias, M.J., Gualillo, O., Kelly, P.A., Dieguez, C., Lago, F., 2004. GH prevents apoptosis in cardiomyocytes cultured in vitro through a calcineurin-dependent mechanism. J. Endocrinol. 180, 325-335.

Gullestad, L., Ueland, T., Vinge, L.E., Finsen, A., Yndestad, A., Aukrust, P., 2012. Inflammatory cytokines in heart failure: mediators and markers. Cardiology 122, 23-35.

Gustafsson, A.B., Brunton, L.L., 2000. Beta-adrenergic stimulation of rat cardiac fibroblasts enhances induction of nitric-oxide synthase by interleukin-1beta via message stabilization. Mol. Pharmacol. 58, 1470-1478.

Hannun, Y.A., Obeid, L.M., 2008. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat. Rev. Mol. Cell. Biol. 9, 139-150.

Haudek, S.B., Taffet, G.E., Schneider, M.D., Mann, D.L., 2007. TNF provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways. J. Clin. Invest. 117, 2692-2701.

Higuchi, Y., Otsu, K., Nishida, K., Hirotani, S., Nakayama, H., Yamaguchi, O., Matsumura, Y., Ueno, H., Tada, M., Hori, M., 2002. Involvement of reactive oxygen species-mediated NF-kappa B activation in TNF-alpha-induced cardiomyocyte hypertrophy. J. Mol. Cell. Cardiol. 34, 233-240.

Hiraoka, E., Kawashima, S., Takahashi, T., Rikitake, Y., Kitamura, T., Ogawa, W., Yokoyama, M., 2001. TNF-alpha induces protein synthesis through PI3-kinase-Akt/PKB pathway in cardiac myocytes. Am. J. Physiol. Heart Circ. Physiol. 280, H1861-1868.

Hu, Y., Li, T., Wang, Y., Li, J., Guo, L., Wu, M., Shan, X., Que, L., Ha, T., Chen, Q., Kelley, J., Li, Y., 2009. Tollip attenuated the hypertrophic response of cardiomyocytes induced by IL-1beta. Front. Biosci. (Landmark Ed.) 14, 2747-2756.

Ing, D.J., Zang, J., Dzau, V.J., Webster, K.A., Bishopric, N.H., 1999. Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. Circ. Res. 84, 21-33.

Jin, L., Wessely, O., Marcusson, E.G., Ivan, C., Calin, G.A., Alahari, S.K., 2013. Prooncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF-alpha in breast cancer. Cancer. Res. 73, 2884-2896.

Jones, S.P., Bolli, R., 2006. The ubiquitous role of nitric oxide in cardioprotection. J. Mol. Cell. Cardiol. 40, 16-23.

Kan, H., Xie, Z., Finkel, M.S., 1999. TNF-alpha enhances cardiac myocyte NO production through MAP kinase-mediated NF-kappaB activation. Am. J. Physiol. 277, H1641-1646.

Kan, W.H., Hsu, J.T., Ba, Z.F., Schwacha, M.G., Chen, J., Choudhry, M.A., Bland, K.I., Chaudry, I.H., 2008. p38 MAPK-dependent eNOS upregulation is critical for 17beta-estradiol-mediated cardioprotection following trauma-hemorrhage. Am. J. Physiol. Heart Circ. Physiol. 294, H2627-2636.

Khurana, S., Hollingsworth, A., Kumar, A., Ross, G.M., Tai, T.C., Piche, M., Venkataraman, K., 2014. Antiapoptotic actions of methyl gallate on neonatal rat cardiac myocytes exposed to H2O2. Oxid. Med. Cell. Longev. 2014, 657512.

Kohler, A.C., Sag, C.M., Maier, L.S., 2014. Reactive oxygen species and excitation-contraction coupling in the context of cardiac pathology. J. Mol. Cell. Cardiol.

Krown, K.A., Page, M.T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K.L., Glembotski, C.C., Quintana, P.J., Sabbadini, R.A., 1996. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. J. Clin. Invest. 98, 2854-2865.

LaPointe, M.C., Sitkins, J.R., 1996. Mechanisms of interleukin-1beta regulation of nitric oxide synthase in cardiac myocytes. Hypertension 27, 709-714.

Li, Q., Guo, Y., Ou, Q., Cui, C., Wu, W.J., Tan, W., Zhu, X., Lanceta, L.B., Sanganalmath, S.K., Dawn, B., Shinmura, K., Rokosh, G.D., Wang, S., Bolli, R., 2009a. Gene transfer

of inducible nitric oxide synthase affords cardioprotection by upregulating heme oxygenase-1 via a nuclear factor-kappa B-dependent pathway. Circulation 120, 1222-1230.

Li, X.Q., Cao, W., Li, T., Zeng, A.G., Hao, L.L., Zhang, X.N., Mei, Q.B., 2009b. Amlodipine inhibits TNF-alpha production and attenuates cardiac dysfunction induced by lipopolysaccharide involving PI3K/Akt pathway. Int. Immunopharmacol. 9, 1032-1041.

Lizano, P., Rashed, E., Kang, H., Dai, H., Sui, X., Yan, L., Qiu, H., Depre, C., 2013. The valosin-containing protein promotes cardiac survival through the inducible isoform of nitric oxide synthase. Cardiovasc. Res. 99, 685-693.

Long, X., Goldenthal, M.J., Wu, G.M., Marin-Garcia, J., 2004. Mitochondrial Ca2+ flux and respiratory enzyme activity decline are early events in cardiomyocyte response to H2O2. J. Mol. Cell. Cardiol. 37, 63-70.

Lu, Y., Zhou, J., Xu, C., Lin, H., Xiao, J., Wang, Z., Yang, B., 2008. JAK/STAT and PI3K/AKT pathways form a mutual transactivation loop and afford resistance to oxidative stress-induced apoptosis in cardiomyocytes. Cell. Physiol. Biochem. 21, 305-314.

Luss, H., Watkins, S.C., Freeswick, P.D., Imro, A.K., Nussler, A.K., Billiar, T.R., Simmons, R.L., del Nido, P.J., McGowan, F.X., Jr., 1995. Characterization of inducible nitric oxide synthase expression in endotoxemic rat cardiac myocytes in vivo and following cytokine exposure in vitro. J. Mol. Cell. Cardiol. 27, 2015-2029.

Markou, T., Makridou, Z., Galatou, E., Lazou, A., 2011. Multiple signalling pathways underlie the protective effect of levosimendan in cardiac myocytes. Eur. J. Pharmacol. 667, 298-305.

Molderings, G.J., Bonisch, H., Bruss, M., Wolf, C., von Kugelgen, I., Gothert, M., 2007. S1P-receptors in PC12 and transfected HEK293 cells: molecular targets of hypotensive imidazoline I(1) receptor ligands. Neurochem. Int. 51, 476-485.

Mukaddam-Daher, S., 2012. An "I" on cardiac hypertrophic remodelling: imidazoline receptors and heart disease. Can. J. Cardiol. 28, 590-598.

Mukaddam-Daher, S., Lambert, C., Gutkowska, J., 1997. Clonidine and ST-91 may activate imidazoline binding sites in the heart to release atrial natriuretic peptide. Hypertension 30, 83-87.

Mukaddam-Daher, S., Menaouar, A., Gutkowska, J., 2006. Receptors involved in moxonidine-stimulated atrial natriuretic peptide release from isolated normotensive rat hearts. Eur. J. Pharmacol. 541, 73-79.

Mukaddam-Daher, S., Menaouar, A., Paquette, P.A., Jankowski, M., Gutkowska, J., Gillis, M.A., Shi, Y.F., Calderone, A., Tardif, J.C., 2009. Hemodynamic and cardiac effects of chronic eprosartan and moxonidine therapy in stroke-prone spontaneously hypertensive rats. Hypertension 53, 775-781.

Munoz, J.P., Chiong, M., Garcia, L., Troncoso, R., Toro, B., Pedrozo, Z., Diaz-Elizondo, J., Salas, D., Parra, V., Nunez, M.T., Hidalgo, C., Lavandero, S., 2010. Iron induces protection and necrosis in cultured cardiomyocytes: Role of reactive oxygen species and nitric oxide. Free Radic. Biol. Med. 48, 526-534.

Paquette, P.A., Duguay, D., El-Ayoubi, R., Menaouar, A., Danalache, B., Gutkowska, J., DeBlois, D., Mukaddam-Daher, S., 2008. Control of left ventricular mass by moxonidine involves reduced DNA synthesis and enhanced DNA fragmentation. Br. J. Pharmacol. 153, 459-467.

Peng, J., Wang, Y.K., Wang, L.G., Yuan, W.J., Su, D.F., Ni, X., Deng, X.M., Wang, W.Z., 2009. Sympathoinhibitory mechanism of moxonidine: role of the inducible nitric oxide synthase in the rostral ventrolateral medulla. Cardiovasc. Res. 84, 283-291.

Piletz, J., Baker, R., Halaris, A., 2008. Platelet imidazoline receptors as state marker of depressive symptomatology. J. Psychiatr. Res. 42, 41-49.

Shindo, T., Ikeda, U., Ohkawa, F., Kawahara, Y., Yokoyama, M., Shimada, K., 1995. Nitric oxide synthesis in cardiac myocytes and fibroblasts by inflammatory cytokines. Cardiovasc. Res. 29, 813-819.

Spiegel, S., Milstien, S., 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat. Rev. Mol. Cell. Biol. 4, 397-407.

Stabile, A.M., Aceros, H., Stockmeyer, K., Abdel Rahman, A.A., Noiseux, N., Mukaddam-Daher, S., 2011. Functional and molecular effects of imidazoline receptor activation in heart failure. Life Sci. 88, 493-503.

Sugden, P.H., Clerk, A., 1998. "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. Circ. Res. 83, 345-352.

Tesfai, J., Crane, L., Baziard-Mouysset, G., Kennedy, W., Edwards, L.P., 2011. Novel I1-imidazoline S43126 enhance insulin action in PC12 cells. Pharmacol. Rep. 63, 1442-1449.

Thaik, C.M., Calderone, A., Takahashi, N., Colucci, W.S., 1995. Interleukin-1 beta modulates the growth and phenotype of neonatal rat cardiac myocytes. J. Clin. Invest. 96, 1093-1099.

Tullio, F., Angotti, C., Perrelli, M.G., Penna, C., Pagliaro, P., 2013. Redox balance and cardioprotection. Basic Res. Cardiol. 108, 392.

Wei, J., Wang, W., Chopra, I., Li, H.F., Dougherty, C.J., Adi, J., Adi, N., Wang, H., Webster, K.A., 2011. c-Jun N-terminal kinase (JNK-1) confers protection against brief but not extended ischemia during acute myocardial infarction. J. Biol. Chem. 286, 13995-14006.

Yndestad, A., Marshall, A.K., Hodgkinson, J.D., Tham el, L., Sugden, P.H., Clerk, A., 2010. Modulation of interleukin signalling and gene expression in cardiac myocytes by endothelin-1. Int. J. Biochem. Cell. Biol. 42, 263-272.

Zhang, J., El-Mas, M.M., Abdel-Rahman, A.A., 2001. Imidazoline I(1) receptor-induced activation of phosphatidylcholine-specific phospholipase C elicits mitogen-activated protein kinase phosphorylation in PC12 cells. Eur. J. Pharmacol. 415, 117-125.

**Figure Captions:**

**Fig. 1.** RT-PCR detection of nischarin product in neonatal rat cardiomyocytes (CM) and fibroblasts (Fb), at first (P1) and second (P2) passages, and after 48h incubation with moxonidine at $10^{-7}$ and $10^{-5}$ M concentrations. Normal mouse C57BL/6 brain and heart were used as positive controls. S18 run in parallel showed that all samples contained equal amounts of cDNA.

**Fig. 2.** Representative bands of nischarin detected by Western blot in cultured neonatal rat ventricular cardiomyocytes and fibroblasts incubated for 24 h with, A: moxonidine $10^{-7}$, $10^{-6}$ , and $10^{-5}$ M, or B: IL-1β and TNF-α, each alone and in combination with $10^{-5}$ M moxonidine. Bar graphs represent ratios of nischarin protein expression compared to GAPDH (loading control) and normalized to corresponding DMEM-incubated cells (100%). n=3-6. **P*<0.05 vs. DMEM; &*P*<0.05 vs. corresponding IL-1β or TNF-α.

**Fig. 3.** Representative bands of phosphorylated Akt and MAPKs (ERK1/2, p38, and JNK) detected by Western blot in cultured neonatal rat ventricular cardiomyocytes incubated for 15 min with moxonidine ($10^{-7}$ and $10^{-5}$ M), without and with the imidazoline $I_1$-receptor antagonist, AGN192403 ($10^{-5}$ M). Bar graphs represent ratios of phosphorylated Akt, ERK1/2, p38, and JNK, compared to GAPDH (loading control) and normalized to corresponding DMEM-incubated cells (100%). n=12. **P*<0.01 vs. DMEM; &*P*<0.05 vs. corresponding moxonidine.

**Fig. 4.** Representative bands of Bax and Bcl2 detected by Western blot in cultured neonatal rat ventricular cardiomyocytes and fibroblasts incubated for 24 h with moxonidine ($10^{-5}$ M). Bar graphs represent Bax, Bcl2, and Bax to Bcl2 ratio, compared to corresponding β-actin (loading control) and normalized to DMEM-incubated control cells (100%). n=6-9. **P*<0.01 vs. DMEM.

**Fig. 5.** Representative bands of phosphorylated Akt and MAPK (ERK1/2, p38, and JNK) detected by Western Blot in cultured neonatal rat ventricular cardiomyocytes incubated for 15 min with IL-1β (5 ng/ml) or TNF-α (10 ng/ml) alone and in combination with $10^{-5}$ M moxonidine. Bar graphs represent ratios of phosphorylated Akt, ERK1/2, p38, and JNK, compared to GAPDH (loading control) and normalized to corresponding DMEM-incubated cells (100%). **P*<0.01 vs. DMEM; &*P*<0.01 vs. corresponding IL-1β or TNF-α, n= 9-12.

**Fig. 6.** Representative bands of phosphorylated Akt and MAPK (ERK1/2, p38, and JNK) detected by Western Blot in cultured neonatal rat ventricular fibroblasts incubated for 15 min with moxonidine ($10^{-5}$ M), IL-1β (5 ng/ml), TNF-α (10 ng/ml), separately and in combination. Bar graphs represent ratios of phosphorylated proteins compared to GAPDH (loading control) and normalized to DMEM-incubated control cells (100%). **P*< 0.01 vs. DMEM; &*P*< 0.01 vs. corresponding IL-1β or TNF-α; n= 9-12.

**Fig. 7.** Representative bands of iNOS protein expression, detected by Western Blot, in cultured neonatal rat ventricular cardiomyocytes and fibroblasts incubated for 24h (left) with DMEM, moxonidine ($10^{-5}$ M), IL-1β (5 ng/ml), TNF-α (10 ng/ml), separately and in combination. LPS, positive control. Right: 48h incubation with DMEM or moxonidine ($10^{-5}$ M). Bar graphs represent ratios of iNOS compared to GAPDH (loading control) and

normalized to corresponding DMEM-incubated cells (100%). *$P$<0.01 vs. DMEM; &$P$<0.05 vs. corresponding IL-1β; n=6-9.

**Fig. 8.** A: Representative flow cytometry after annexin V ($x$-axis) and propidium iodide (PI, $y$-axis) staining in cardiomyocytes after 48 h incubation in starvation conditions (DMEM) and moxonidine ($10^{-7}$ and $10^{-5}$ M), without and with and imidazoline $I_1$-receptor antagonist, AGN192403 ($10^{-5}$ M). DMEM+10%FBS, normal growth control; $H_2O_2$ ($10^{-4}$ M) death control. B: Bar graphs represent percent cardiomyocytes in early apoptosis (annexin positive, PI negative), necrosis (PI positive) and alive (annexin negative, PI negative). Data presented as percent corresponding DMEM. *$P$<0.01 vs. DMEM; &$P$<0.05 vs. corresponding moxonidine; \$$P$<0.05 vs. $H_2O_2$; n= 9-12.

**Fig. 9.** MTT assay in cardiomyocytes incubated for 48h in starvation conditions (DMEM) without and with moxonidine ($10^{-5}$ M), IL-1β (5 ng/ml), TNF-α (10 ng/ml), $H_2O_2$ ($10^{-4}$ M), separately and in combination. *$P$<0.05 vs. DMEM; &$P$<0.05 vs. corresponding IL-1β, TNF-α, or $H_2O_2$; n=12-15.

**Fig. 10.** A: Representative fluorescent micrographs of calcein (Green) and ethidium homodimer (Red) staining in cardiomyocytes incubated for 48 h in starvation condition (DMEM) without and with moxonidine ($10^{-5}$ M), IL-1β (5 ng/ml), or TNF-α (10 ng/ml), separately and in combination; n=3. Scale bar: 200μm. B: Representative fluorescent micrographs and corresponding bar graphs of ethidium homodimer staining in cardiomyocyte incubated for 48 h in starvation condition (DMEM) without and with moxonidine ($10^{-5}$ M), and imidazoline $I_1$-receptor antagonist AGN192403 ($10^{-5}$ M), and protein inhibitors, separately and in combination. SB (10 μM), p38 inhibitor; SP (10 μM), JNK inhibitor; SMT (1 μM),

iNOS inhibitor. *$P$<0.05 vs. DMEM; &$P$<0.05 vs. corresponding inhibitor; n=3; Scale bar: 200μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

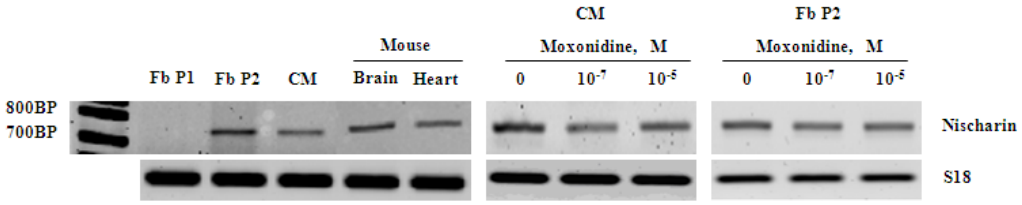
**Fig. 11.** A: Representative fluorescent micrographs and corresponding bar graphs of ethidium homodimer staining in cardiomyocyte incubated for 48 h in starvation condition (DMEM) with IL-1β (5 ng/ml), without and with moxonidine ($10^{-5}$ M) and signalling protein inhibitors. *$P$<0.05 vs. DMEM; &$P$<0.05 vs. corresponding IL-1β; #$P$<0.05 vs. corresponding IL-1β+moxonidine; $$P$<0.05 vs. corresponding inhibitor; n=3. B: Representative fluorescent micrographs and corresponding bar graphs of ethidium homodimer staining in cardiomyocyte incubated for 48 h in starvation condition (DMEM) with TNF-α (10 ng/ml), without and with moxonidine ($10^{-5}$ M) and signalling protein inhibitors. *$P$<0.05 vs. DMEM; &$P$<0.05 vs. corresponding cytokine; #$P$<0.05 vs. corresponding TNF+moxonidine; $$P$<0.05 vs. corresponding inhibitor; n=3. PD (10 μM), ERK inhibitor; SB (10 μM), p38 inhibitor; SMT (1 μM), iNOS inhibitor; wortmannin (0.5 μM), PI3K/Akt inhibitor. Scale bar: 200μm.

**Fig. 12.** A: Representative fluorescent micrographs of calcein (green) staining and corresponding bar graphs of fibroblasts after 48 h incubation with moxonidine ($10^{-5}$ M), IL-1β (5ng/ml), or TNF-α (10 ng/ml), separately and in combination in serum starvation conditions (DMEM). B: Representative fluorescent micrographs of ethidium homodimer (red) staining and corresponding bar graphs of fibroblasts after 48 h incubation with moxonidine ($10^{-5}$ M), IL-1β (5ng/ml), or TNF-α (10 ng/ml), separately and in combination in serum starvation conditions (DMEM). *$P$<0.05 vs. DMEM; &$P$<0.05 vs. corresponding cytokine; n=3. Scale

bar: 200μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 13.** Schematic diagram showing hypothetical mechanisms of cardiomyocyte protection by moxonidine in the presence of cytokines. IL-1β alone increases p38, JNK, and iNOS and results in cell death. Moxonidine causes an increase in the anti-apoptotic protein Bcl2 and reduces the apoptotic protein Bax, favoring cardiomyocyte survival. Moxonidine also potentiates IL-1β-induced p38, JNK and iNOS. Once iNOS is expressed, it produces large quantities of nitric oxide (NO), which can be both pro- and anti-apoptotic, depending on quantities and downstream signalling pathways: NO combining with oxidants generates the free radicals, peroxynitrites (ONOO-), inhibits mitochondrial enzymes, and induces cell death. On the other hand, NO can be cytoprotective, inducing Bcl2, which normally protects cells from apoptotic triggers. Through activation of the pro-survival/anti-apoptotic signals, as well as through its antioxidant effect, moxonidine may shift the balance towards the anti-apoptotic effect of NO. Opposite mechanisms may take place in fibroblasts, where moxonidine increases Bax, reduces basal and IL-1β-induced JNK and iNOS and does not exert an anti-oxidant effect, thus the balance is shifted towards cell death. SB, p38 inhibitor; SP, JNK inhibitor; SMT, iNOS inhibitor. Solid lines, potentiation; Dotted lines, inhibition.

Figure 1



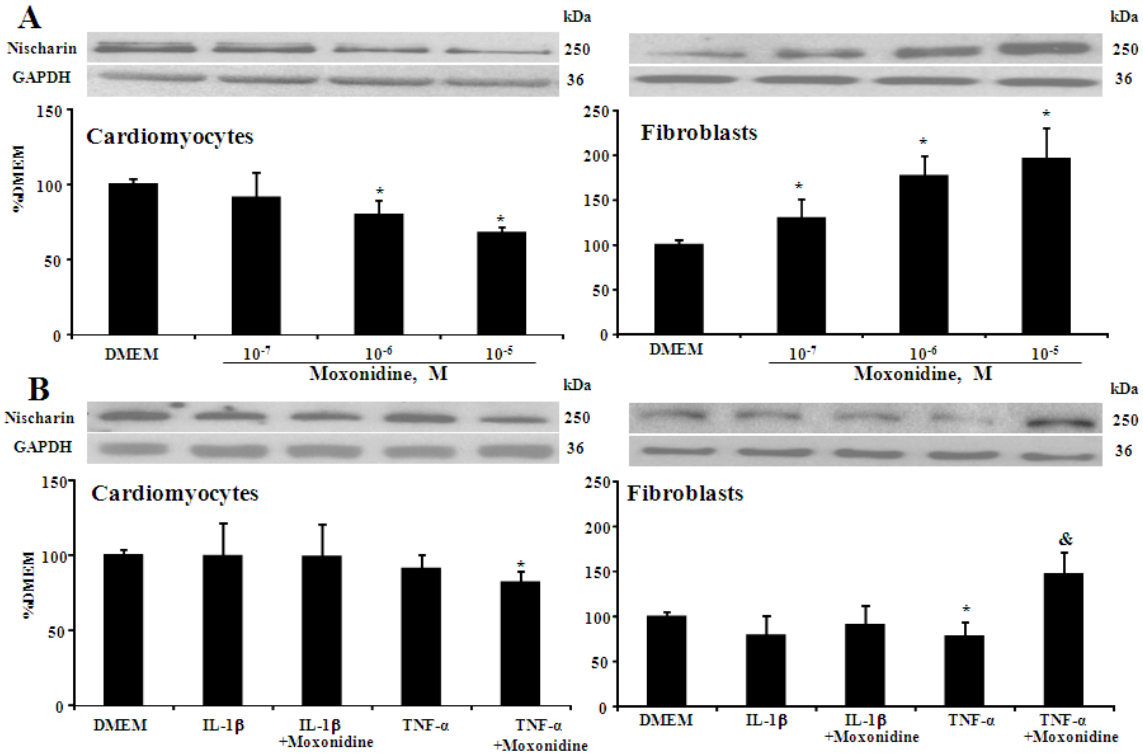




Figure 2

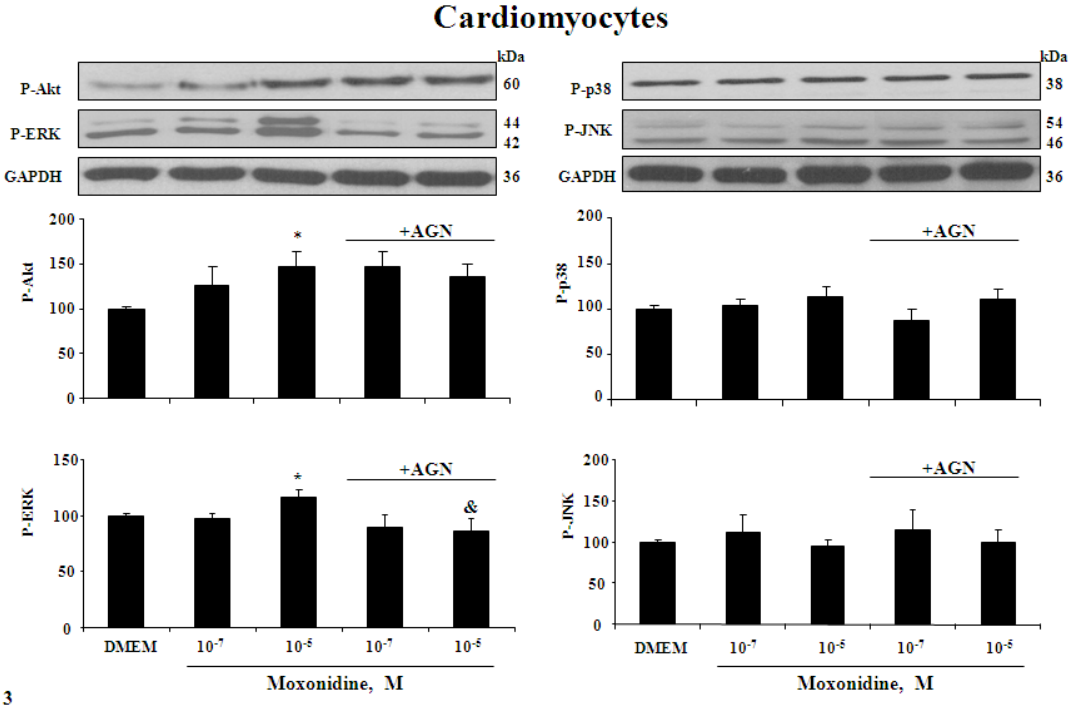
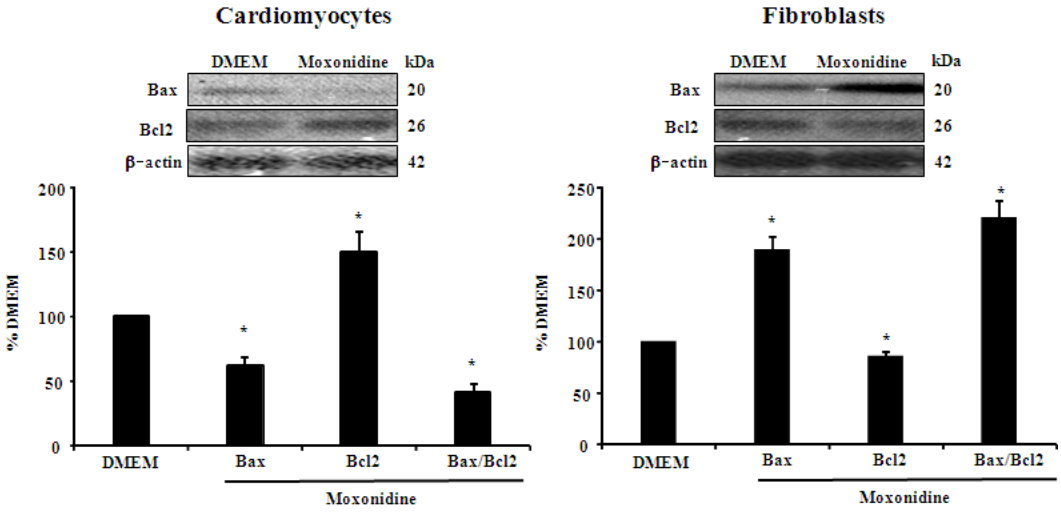



Figure 3

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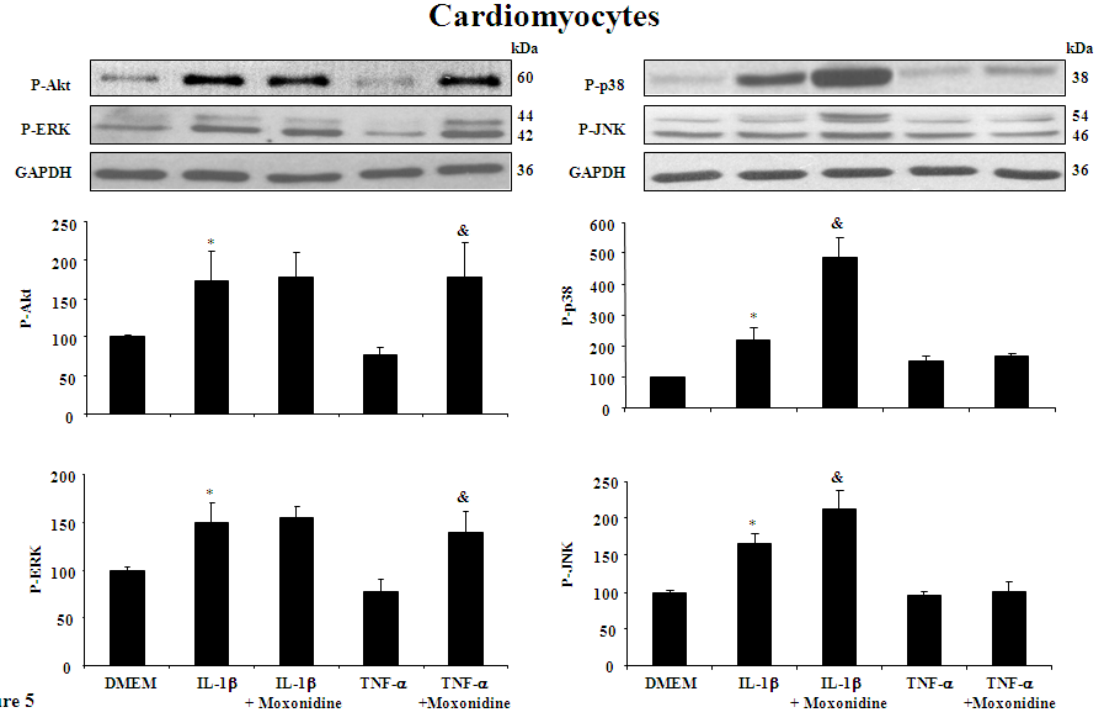



Figure 5

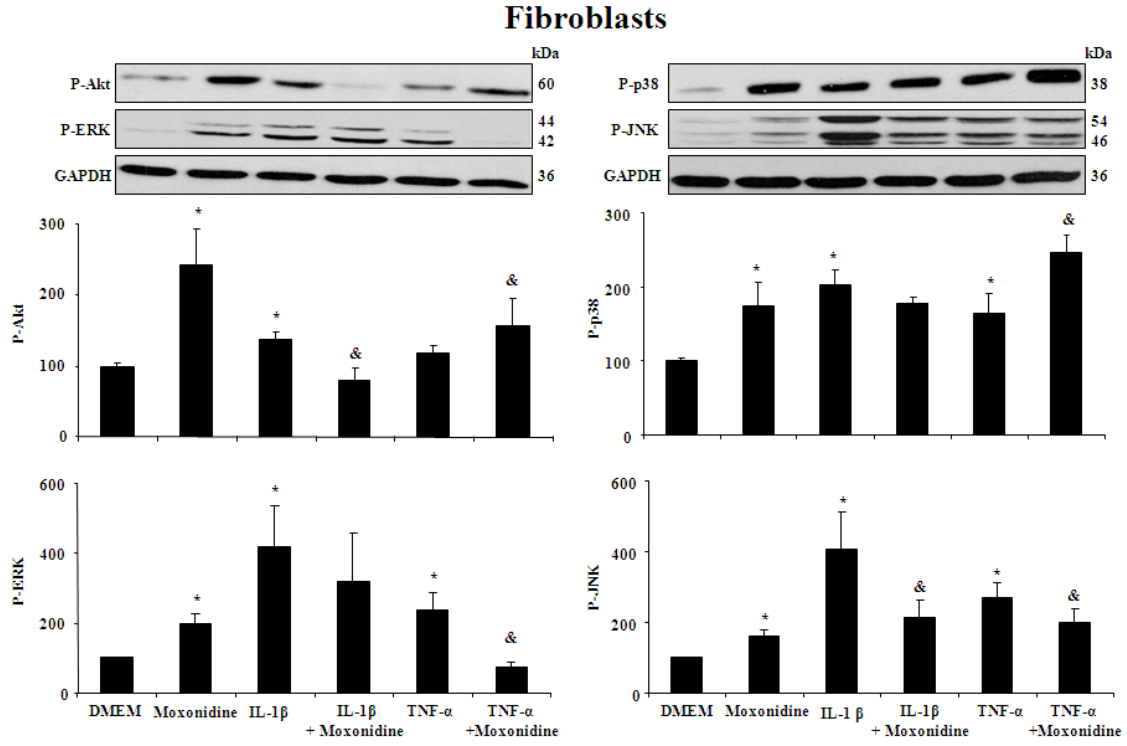



Figure 6

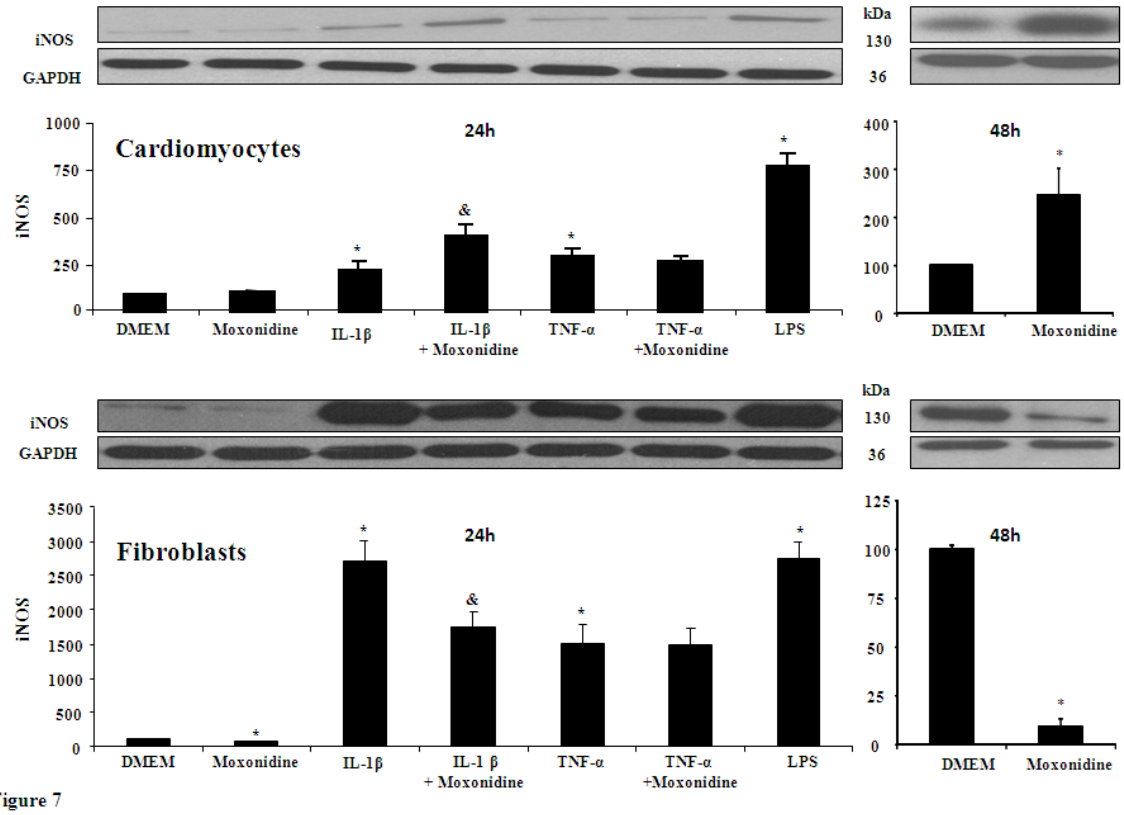

iNOS
GAPDH
kDa
130
36
Cardiomyocytes
24h
48h
iNOS
1000
750
500
250
0
400
300
200
100
0
DMEM
Moxonidine
IL-1β
IL-1β + Moxonidine
TNF-α
TNF-α +Moxonidine
LPS
iNOS
GAPDH
kDa
130
36
Fibroblasts
24h
48h
iNOS
3500
3000
2500
2000
1500
1000
500
0
125
100
75
50
25
0
DMEM
Moxonidine
IL-1β
IL-1 β + Moxonidine
TNF-α
TNF-α +Moxonidine
LPS

**Figure 7**

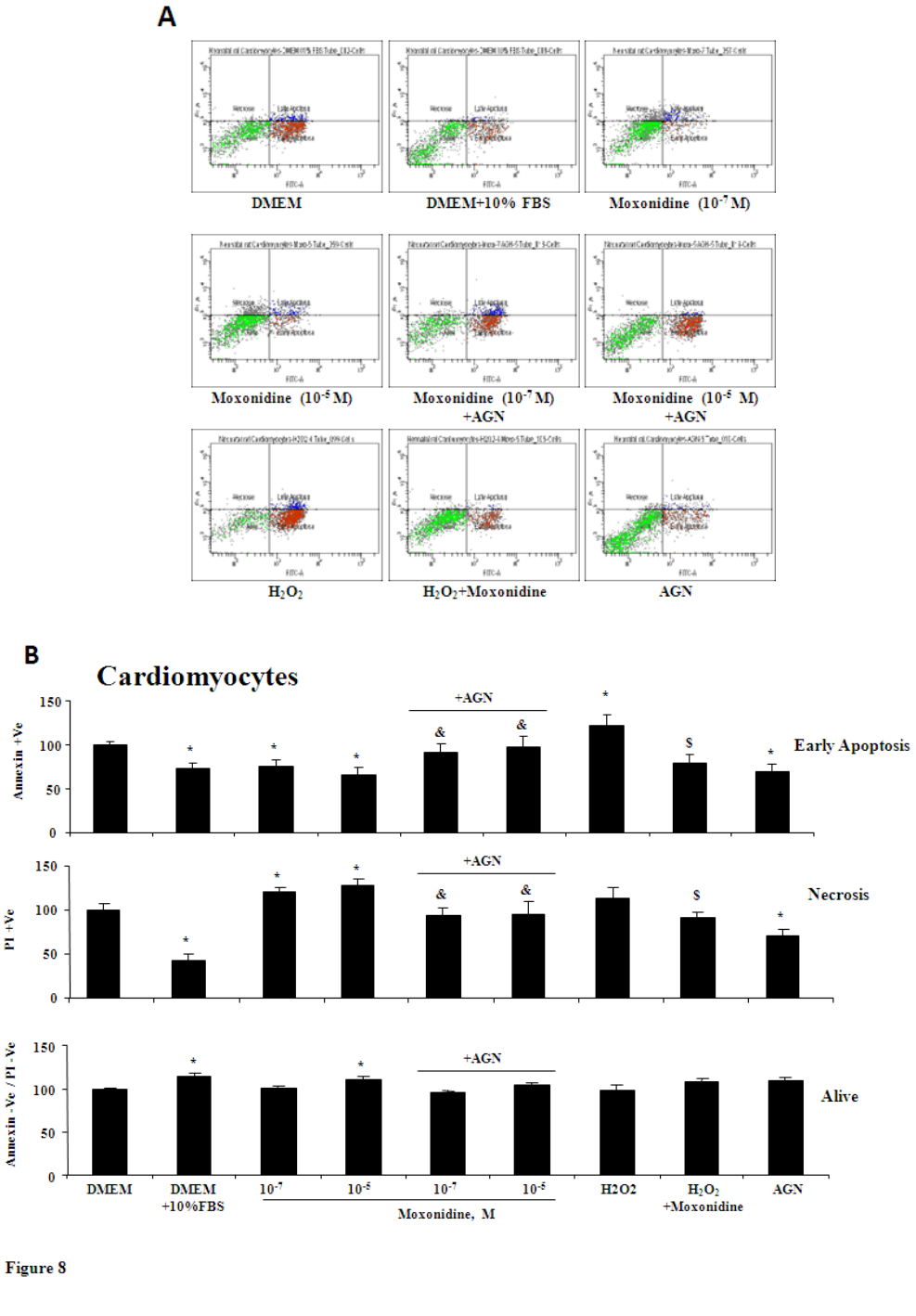

A
DMEM
DMEM+10% FBS
Moxonidine (10-7 M)
Moxonidine (10-5 M)
Moxonidine (10-7 M) +AGN
Moxonidine (10-5 M) +AGN
H2O2
H2O2+Moxonidine
AGN
B
Cardiomyocytes
Annexin +Ve
PI +Ve
Annexin -Ve / PI -Ve
+AGN
Early Apoptosis
Necrosis
Alive
DMEM
DMEM +10%FBS
10-7
10-5
Moxonidine, M
H2O2
H2O2 +Moxonidine
AGN

Figure 8

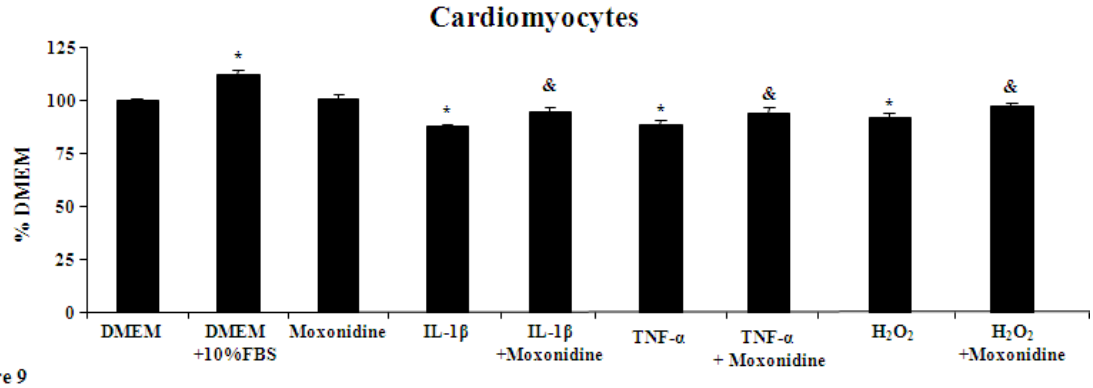
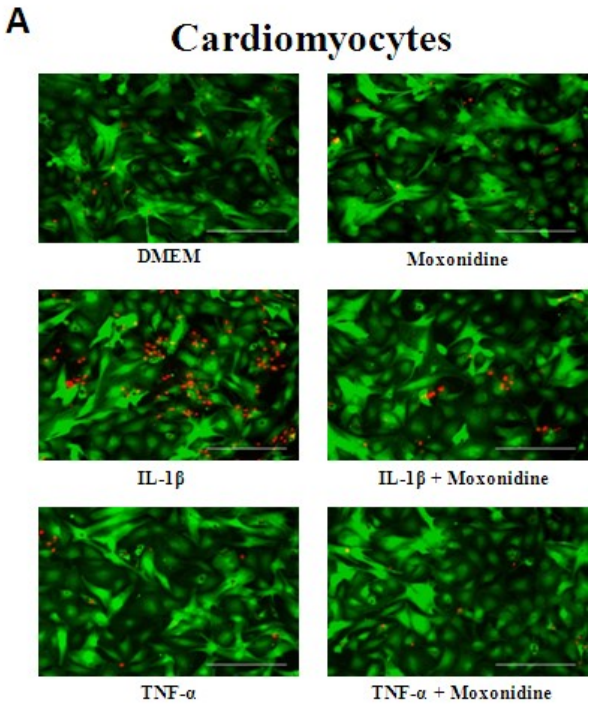

Cardiomyocytes
% DMEM
125
100
75
50
25
0
*
*
&
*
&
*
&
DMEM
DMEM +10%FBS
Moxonidine
IL-1β
IL-1β +Moxonidine
TNF-α
TNF-α + Moxonidine
$H_2O_2$
$H_2O_2$ +Moxonidine

Figure 9


A
Cardiomyocytes
DMEM
Moxonidine
IL-1β
IL-1β + Moxonidine
TNF-α
TNF-α + Moxonidine

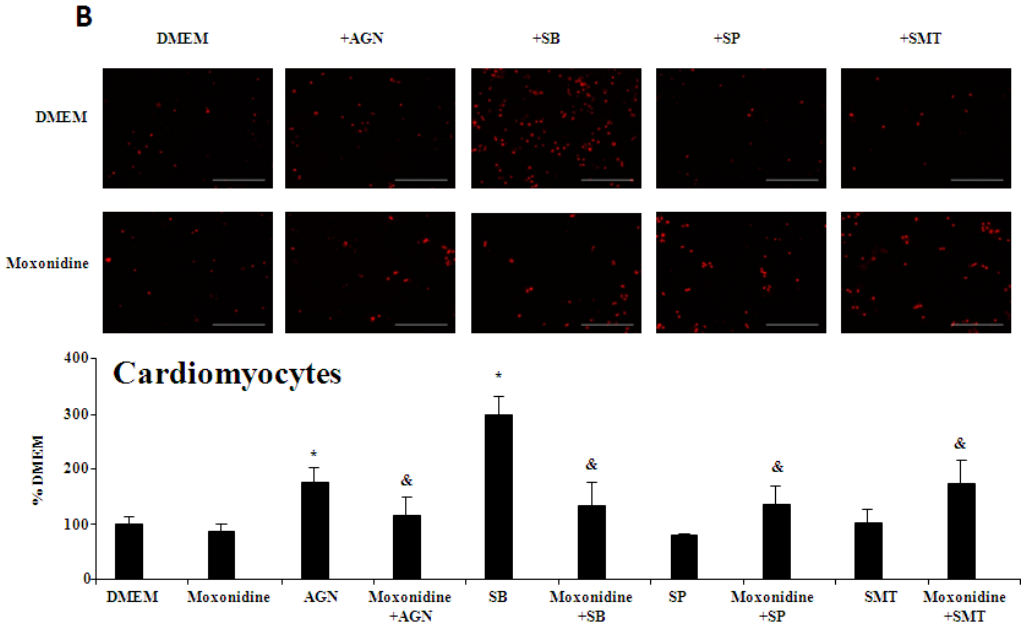
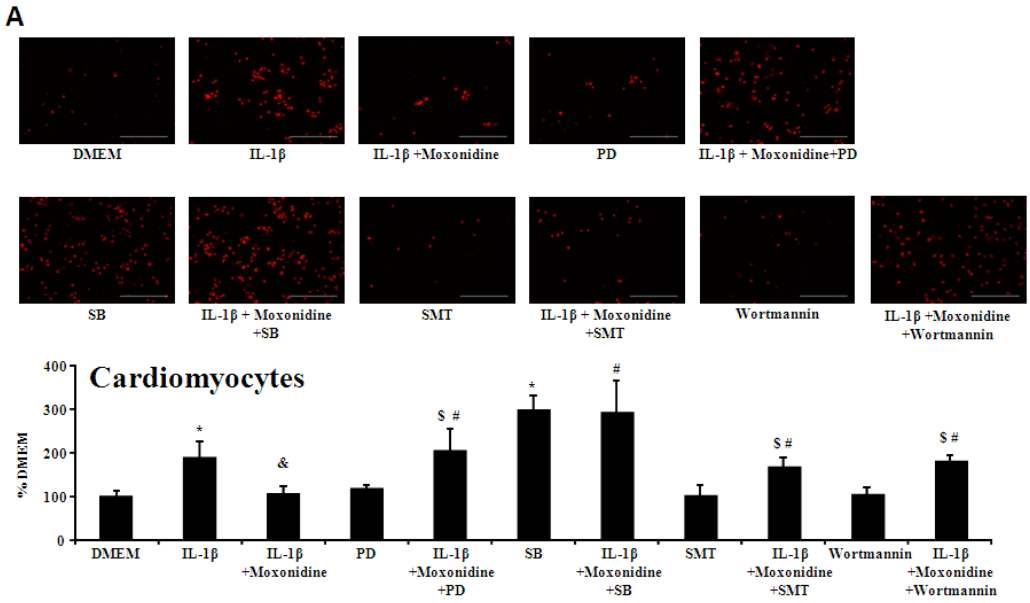

B
DMEM
+AGN
+SB
+SP
+SMT
DMEM
Moxonidine
Cardiomyocytes
% DMEM
400
300
200
100
0
*
*
&
&
&
&
DMEM
Moxonidine
AGN
Moxonidine +AGN
SB
Moxonidine +SB
SP
Moxonidine +SP
SMT
Moxonidine +SMT

Figure 10


A
DMEM
IL-1β
IL-1β +Moxonidine
PD
IL-1β + Moxonidine+PD
SB
IL-1β + Moxonidine +SB
SMT
IL-1β + Moxonidine +SMT
Wortmannin
IL-1β +Moxonidine +Wortmannin
Cardiomyocytes
% DMEM
400
300
200
100
0
*
&
$ #
*
#
$ #
$ #
DMEM
IL-1β
IL-1β +Moxonidine
PD
IL-1β +Moxonidine +PD
SB
IL-1β +Moxonidine +SB
SMT
IL-1β +Moxonidine +SMT
Wortmannin
IL-1β +Moxonidine +Wortmannin

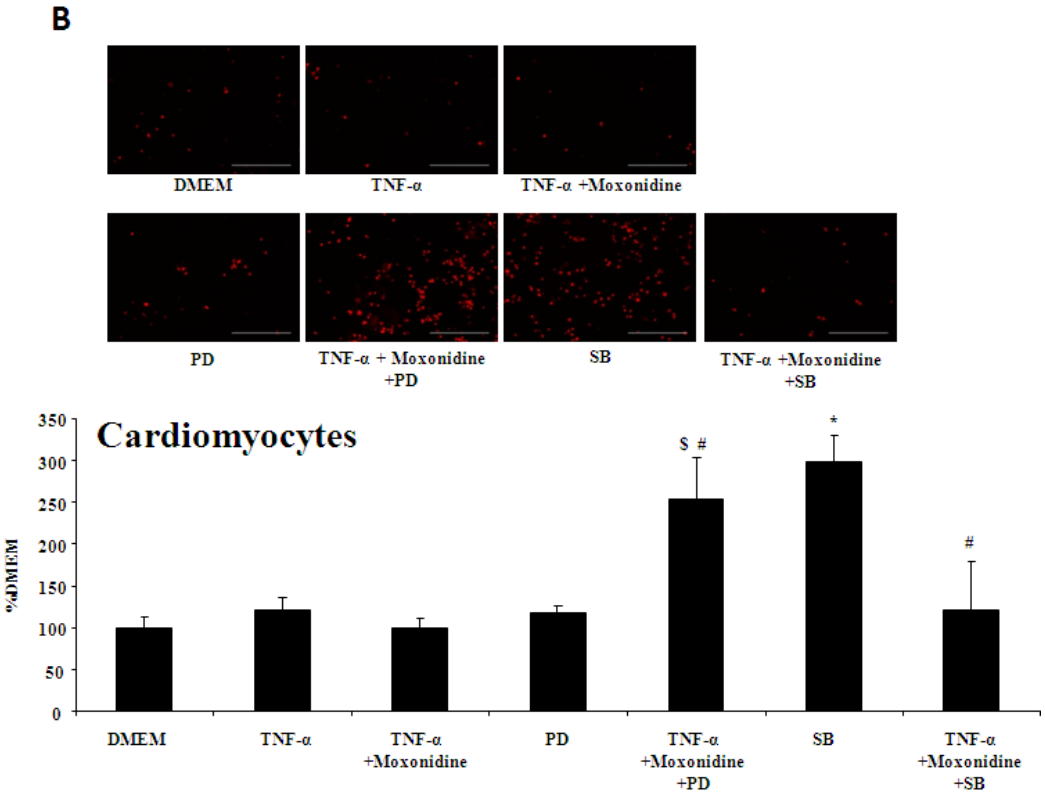
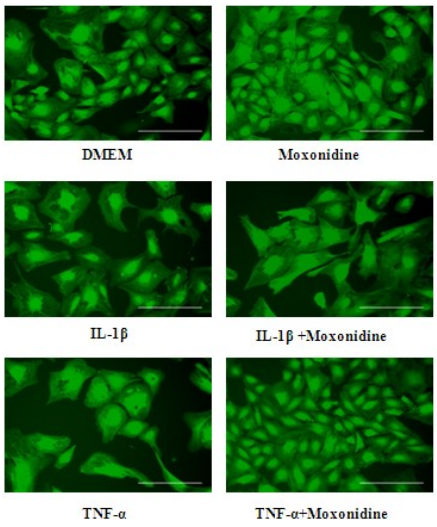
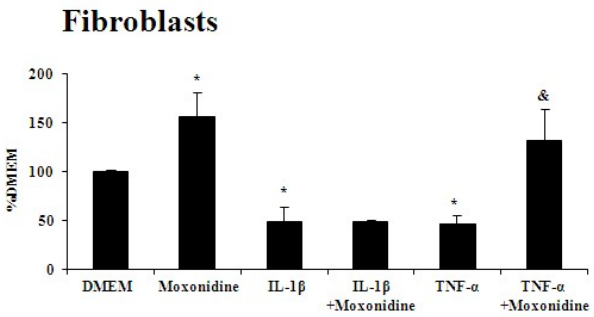

B
DMEM
TNF-α
TNF-α +Moxonidine
PD
TNF-α + Moxonidine +PD
SB
TNF-α +Moxonidine +SB
Cardiomyocytes
%DMEM
350
300
250
200
150
100
50
0
$ #
*
#
DMEM
TNF-α
TNF-α +Moxonidine
PD
TNF-α +Moxonidine +PD
SB
TNF-α +Moxonidine +SB

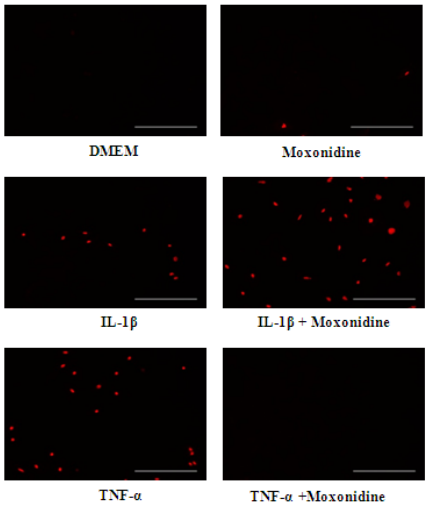
**Figure 11**

A


DMEM
Moxonidine
IL-1β
IL-1β +Moxonidine
TNF-α
TNF-α+Moxonidine


Fibroblasts
%DMEM
200
150
100
50
0
*
*
*
&
DMEM
Moxonidine
IL-1β
IL-1β +Moxonidine
TNF-α
TNF-α +Moxonidine

B


DMEM
Moxonidine
IL-1β
IL-1β + Moxonidine
TNF-α
TNF-α +Moxonidine

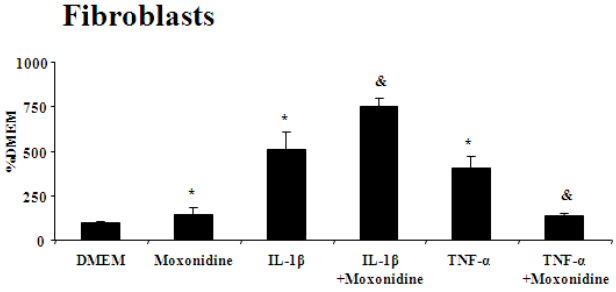

Fibroblasts
%DMEM
1000
750
500
250
0
*
*
*
&
&
DMEM
Moxonidine
IL-1β
IL-1β +Moxonidine
TNF-α
TNF-α +Moxonidine

Figure 12

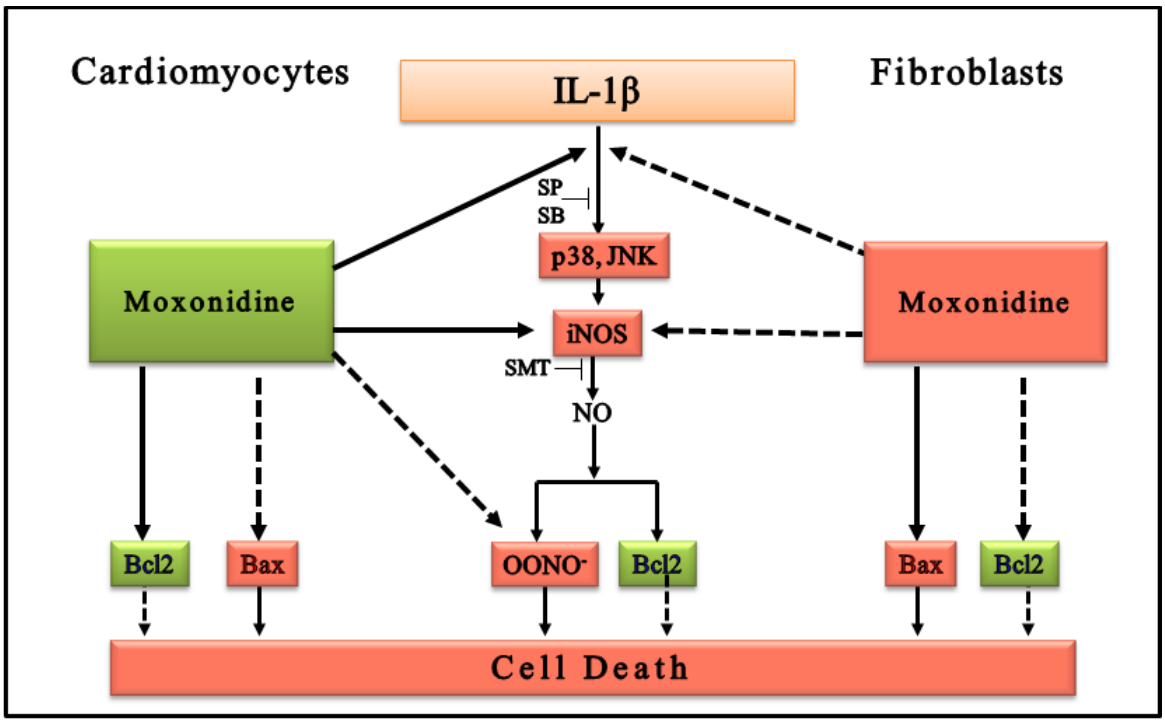

Cardiomyocytes
IL-1β
Fibroblasts
SP
SB
p38, JNK
Moxonidine
iNOS
Moxonidine
SMT
NO
Bcl2
Bax
OONO-
Bcl2
Bax
Bcl2
Cell Death

Figure 13

## 3.6 Supplementary data:

This small section pertains to data obtained in the framework of the studies, but not used in the publications. These data do not change the papers' conclusions, but allow for a more complete discussion in this thesis.

**Activation of the imidazoline $I_1$-receptor by moxonidine in neonatal rat cardiomyocytes induces the release of atrial natriuretic peptide (ANP).**

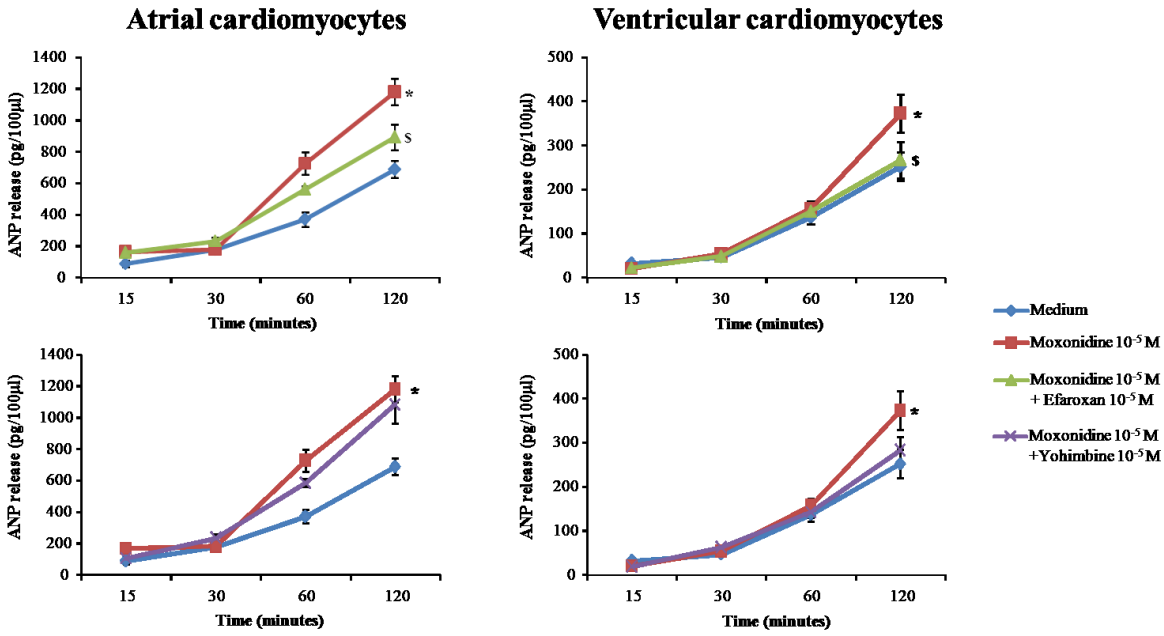



Figure 14: Time-dependent ANP release to the culture medium (DMEM) from cultured neonatal rat cardiomyocytes following incubation with the imidazoline I1-receptor agonist moxonidine (10-5 M), alone or in combination with the imidazoline I1-receptor antagonist efaroxan (10-5 M) or the α2-adrenergic receptor antagonist yohimbine (10-5 M). * P<0.01 vs. medium; $ P<0.01 vs. moxonidine.

Cardiomyocytes were incubated in 1 ml DMEM or DMEM+moxonidine for 120 minutes. Aliquots (50 µl) were collected at indicated times (0, 15, 30, 60 and 120 minutes) for measurement of ANP, by radioimmunoassay. Compared to DMEM alone, moxonidine significantly stimulated the release of ANP at 60 and 120 min in atrial and ventricular cardiomyocytes, respectively. Moxonidine-induced ANP release was inhibited by co-incubation with efaroxan more than by yohimbine. The greater effect of efaroxan compared to

the α2-adrenergic receptor antagonist yohimbine suggests a predominant involvement of imidazoline $I_1$-receptors in the action. Most importantly, these studies indicate that cardiac imidazoline $I_1$-receptors are functional without CNS contribution.

**Activation of the imidazoline $I_1$-receptor by moxonidine induces the release of atrial natriuretic peptide (ANP) form hearts isolated from normotensive (Sprague-Dawley) and hypertensive (SHR) rats. The effect of moxonidine is potentiated in SHR hearts.**

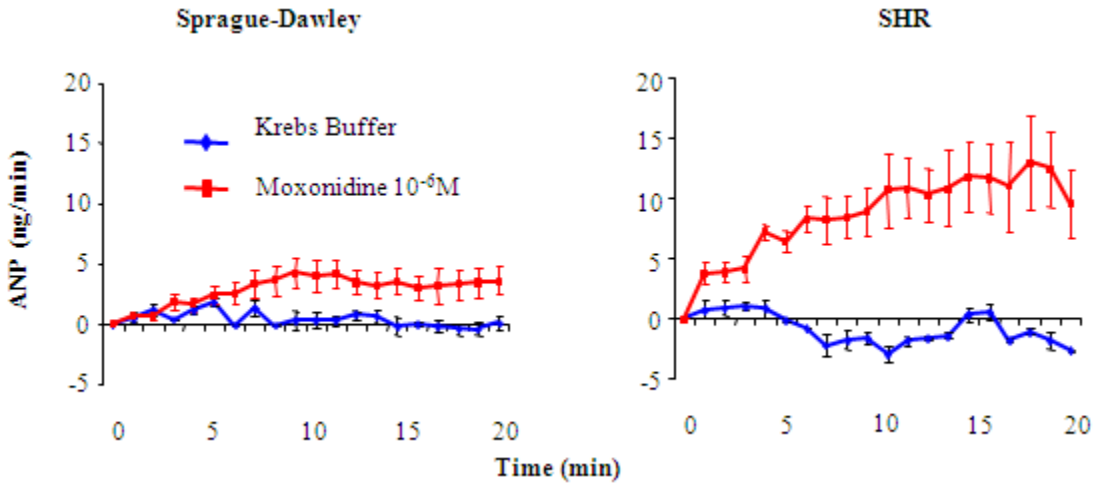



Figure 15 : ANP release following perfusion of isolated rat hearts with the imidazoline $I_1$-receptor agonist moxonidine ($10^{-6}$ M) or control Krebs buffer.

After 30 min equilibration, isolated rat hearts were perfused *ex vivo* with moxonidine ($10^{-6}$ M) or Krebs-Henseleit perfusion buffer for 20 minutes. Aliquots were collected every minute for the measurement of ANP, by radioimmunoassay. ANP concentration was corrected for the flow per minute and reported as ng/min. Compared to buffer perfusion, moxonidine significantly stimulated the release of ANP in normotensive hearts; this effect was augmented in hypertensive SHR hearts. These studies indicate that cardiac imidazoline $I_1$-receptors are functional without CNS contribution and are consistent with the notion that imidazoline $I_1$-receptors are altered in cardiovascular diseases.

# Chapter 4. Discussion

Previous studies from this laboratory have shown that chronic activation of the imidazoline $I_1$-receptor with the selective agonist moxonidine regresses left ventricular hypertrophy (LVH) in hypertensive rats, at both hypotensive and sub-hypotensive doses (Mukaddam-Daher et al., 2009; Paquette et al., 2008). Regression of LVH is associated with a sustained reduction of DNA synthesis and transient stimulation of DNA fragmentation and apoptotic proteins Bax and caspase 3, occurring after 1 week but subsiding by 4 weeks of treatment (Paquette et al., 2008). Cardiomyocyte apoptosis plays a causal role in the development of heart failure through progressive left ventricular wall thinning (Kemp & Conte, 2012; Liao et al., 2004; Ward et al., 2011) and inhibition of cardiomyocyte apoptosis attenuates contractile dysfunction in heart failure (Foo, Mani, & Kitsis, 2005). Accordingly, if moxonidine-stimulated apoptosis occurred in cardiomyocytes, cardiac dysfunction would ensue, despite the transient increase in ventricular ANP synthesis that also subsided by 4 weeks of treatment (El-Ayoubi et al., 2003), indicating treatment efficacy. Taken in the context of the negative results of the MOXCON trial in heart failure patients (Cohn et al., 2003), these studies led us to question the effect of moxonidine on cardiac function. Accordingly, studies investigated in hypertensive and heart failure animals the dose- and time-dependent effects of moxonidine on cardiac function. In addition, studies investigated the effect of moxonidine on hypertension- and heart failure-associated mechanisms, specifically cytokines.

The models chosen in these studies, the 12-week old spontaneously hypertensive rat (SHR) and the 6 month-old BIO14.6 hamster (Aceros et al., 2011; Stabile et al., 2011), are well described models of hypertensive and normotensive hypertrophic cardiomyopathy, respectively. They share an increased sympathetic activity, LVH, and a decreased diastolic function (Aceros et al., 2011; Cabassi et al., 2007; Gertz, 1972; Okamoto & Aoki, 1963; Slama, Ahn, Varagic, Susic, & Frohlich, 2004; Sole, Lo, Laird, Sonnenblick, & Wurtman, 1975; Stabile et al., 2011). However, the SHR differs from BIO14.6 hamsters in that systolic function is preserved in SHR (Slama et al., 2004), while in the BIO14.6 hamster systolic function declines in an age-dependent manner until ventricular dilatation and overt heart failure occur at the age of 10 months and above. This age-dependent development of heart

failure has been shown in previous studies (Gertz, 1972; Kyoi et al., 2006; Strobeck et al., 1979), as well as in our present results (Stabile et al., 2011).

In addition, the SHR presents with a robust cardiac inflammatory response, shown by an increase in serum pro-inflammatory markers (Aceros et al., 2011). On the other hand, serum pro-inflammatory markers in the young BIO14.6 cardiomyopathic hamsters are not significantly different from normal controls, but continuous low grade inflammation is observed in the heart, which manifests in the older hamsters (Gertz, 1972; Stabile et al., 2011). These characteristics are similar to those observed in humans suffering from chronic hypertension and dilated cardiomyopathy, respectively (Dornas & Silva, 2011; Houser et al., 2012), allowing for evaluation of the imidazoline $I_1$-receptor activation by moxonidine under chronic conditions, mimicking the evolution from hypertension, to heart failure, and finally to the state of uncompensated dilated heart failure, observed in untreated patients (Nielsen, 1986; Strauer, 1984).

## 4.1 Moxonidine improves cardiac function in hypertensive and heart failure models, in association with anti-inflammatory and anti-oxidative actions

Heart rate is regulated by the balance between sympathetic (tachycardic) and parasympathetic (bradycardic) actions at the level of the sinoatrial node in response to cardiovascular stress (Tobaldini et al., 2013). Increased heart rate (above 90 bpm) has been related to increase in cardiovascular and all-cause mortality (Kannel, Kannel, Paffenbarger, & Cupples, 1987; Kristal-Boneh, Silber, Harari, & Froom, 2000), and decreasing heart rate has been independently related to a decrease in mortality in coronary artery disease (Fox, Ford, Steg, Tendera, & Ferrari, 2008) and heart failure (Bohm et al., 2010). Our studies have shown that high concentrations of moxonidine reduce heart rate in both SHR and BIO14.6 hamsters (Aceros et al., 2011; Stabile et al., 2011), suggesting a possible beneficial effect of long term treatment. This decrease might be related to a centrally mediated decrease in sympathetic output by moxonidine acting on imidazoline $I_1$-receptors located on the rostro-ventro lateral

medulla (Chan et al., 2005; Haxhiu, Dreshaj, Schafer, & Ernsberger, 1994). On the other hand, in the SHR, heart rate was decreased by both concentrations, despite that the low concentration of moxonidine did not reduce blood pressure (Aceros et al., 2011). The selective decrease in heart rate by low moxonidine concentration is consistent with previous studies from our laboratory that have shown that moxonidine reduces heart rate in isolated rat hearts (ex vivo) by mechanisms that include imidazoline $I_1$-receptors in the heart (Mukaddam-Daher et al., 2006). Schafer et al., (2003) have also shown that moxonidine reduces norepinephrine release induced by stimulation of epicardial ganglia on isolated rat hearts (Schafer et al., 2003). Additionally, it has been described that moxonidine has parasympathomimetic actions in normotensive animals and humans (Kaya, Barutcu, Esen, Celik, & Onrat, 2010; Turcani, 2008), an effect that might contribute to the decrease in heart rate independent of changes in blood pressure. Finally, activation of the imidazoline $I_1$-receptor by moxonidine induces ANP release in isolated hearts (Mukaddam-Daher et al., 2006), an effect particularly important in hypertensive animals (Figure 15). ANP potentiates reflex bradycardia via guanylyl cyclase receptors, probably by inducing NO and cGMP at cardiac level (Thomas & Woods, 2003), possibly leading to an increase sensitivity to bradycardic stimuli. Taken together, these studies imply that the bradycardia observed in SHR treated with non-hypotensive concentration of moxonidine results from a combined action on central and peripheral imidazoline $I_1$-receptors.

Consistent with previous reports (Slama et al., 2004), our echocardiographic measurements confirm the presence of diastolic dysfunction and the absence of systolic dysfunction in untreated SHR. The measurements also reveal that activation of imidazoline $I_1$-receptors by moxonidine regresses LVH and improves diastolic and global cardiac function without negatively affecting systolic function (Aceros et al., 2011). Furthermore, moxonidine improves diastolic dysfunction and prevents the time-dependent deterioration in systolic function in young hamsters with moderate heart failure, as well as older hamsters with advanced heart failure. The effect is particularly important in the younger group, where treatment reduces end systolic and end diastolic volumes, pointing towards an improvement in ventricular geometry following treatment (Stabile et al., 2011).

Diastolic and systolic dysfunctions are considered as different evolutionary stages of heart failure (Hunt et al., 2009). Diastolic dysfunction is an alteration commonly observed in hypertensive patients (Palatini et al., 2001; Slama, Susic, Varagic, & Frohlich, 2002), is associated with normal ejection fraction. Patients with symptomatic heart failure usually have both diastolic and systolic dysfunction, defined as ejection fraction below 55% (Lang et al., 2005). Also, in around 40% of diabetic patients, diastolic dysfunction without other obvious causes (like ischemia or hypertension) has been found, configuring the first phases of diabetic cardiomyopathy (Pappachan, Varughese, Sriraman, & Arunagirinathan, 2013; Schilling & Mann, 2012).

An increase in mortality has been observed in patients with moderate and severe diastolic dysfunction (Halley, Houghtaling, Khalil, Thomas, & Jaber, 2011); and diastolic dysfunction increases the risk of symptomatic heart failure by 30% (Lam et al., 2011).

Diastolic dysfunction can be defined as "the inability of the heart to fill with blood during diastole" (Slama et al., 2002). It results from impaired LV relaxation and an increase in LV wall stiffness, both of which impede blood flow into the ventricle. Thus, it is related to ventricular fibrosis, hypertrophy, and alterations in cardiomyocyte relaxation (Slama et al., 2002; Wan, Vogel, & Chen, 2014), characteristics also observed in the SHR where left ventricular relaxation is impaired and stiffness increased (Slama et al., 2004).

The quality of diastole is affected by the static properties of the myocardium, the extracellular matrix (ECM) and the cardiomyocytes and their interaction via matricellular proteins (Wan et al., 2014). The ECM stiffness is determined by the amount of collagen, the abundance of collagen I, and collagen cross linking (Bonow et al., 2012; Diez, 2008; Diez et al., 2002). Severe diastolic dysfunction with an increased collagen deposition when compared with normoglycemic rats is described in 18 week old type II diabetic Goto-Kakizaki rats (D'Souza et al., 2014; El-Omar, Yang, Phillips, & Shah, 2004) and treatment with the hypoglycemic medication metformin diminishes cardiac fibrosis and ventricular hypertrophy, and improves diastolic function (Picatoste et al., 2013). In Dahl salt-sensitive rats, a model of accelerated hypertensive diastolic failure, an increase in collagen deposition is also observed. Treatment of these rats with sub-hypotensive doses of the calcium channel antagonist nifedipine diminishes collagen deposition and ventricular hypertrophy, and improves diastolic function (Yamada et

al., 2009). These studies highlight the importance of blood pressure and glucose metabolism in the control of cardiac function, but most importantly, suggest that reducing collagen deposition and ventricular hypertrophy improves diastolic function and that the effect is independent of changes in blood pressure.

On the other hand, systolic function is influenced by changes in the relative abundance of collagen I and collagen III. A reduced pro-collagen I to pro-collagen III ratio was observed in patients with idiopathic dilated cardiomyopathy when compared to normal controls (Timonen et al., 2008) and a higher ratio was found in patients successfully treated with a left ventricular assist device compared to those that required heart transplantation (Liang et al., 2004). These studies demonstrate that a change in collagen I to collagen III ratio favouring a decrease in collagen I may be deleterious in heart failure.

Consistent with the above studies, our studies reveal that moxonidine treatment reduces both cardiomyocyte hypertrophy and ventricular collagen deposition in SHR and cardiomyopathic hamsters. Moxonidine administration to young cardiomyopathic hamsters also increased the relative content of collagen I and decreased relative content of collagen III, an effect that may hinder further ventricular dilatation, improving ventricular geometry (Stabile et al., 2011). The decreased cardiomyocyte hypertrophy, combined with a decrease in collagen deposition and a relative increase in collagen type I expression could explain, in part, the observed improvement in both diastolic (SHR and cardiomyopathic hamsters) and systolic (cardiomyopathic hamsters) functions.

In addition to collagen deposition, intrinsic cardiomyocyte stiffness also contributes to diastolic LV dysfunction (Wan et al., 2014). The extent of ventricular hypertrophy is directly related to the level of diastolic dysfunction observed in patients with hypertrophic cardiomyopathy or hypertension (De Marchi, Allemann, & Seiler, 2000). It has been observed that cardiomyocytes from patients with diastolic heart failure have developed hypertrophy and increased passive stiffness (van Heerebeek et al., 2006). Elevation of cardiomyocyte stiffness has been related to the cytoskeletal protein titin isoform switching, phosphorylation status, as well as oxidative stress-induced formation of disulfide bridges within the titin molecule (Borbely et al., 2009; Borbely et al., 2005; Borlaug & Paulus, 2011). The mechanisms include the activation of protein kinase A, a pathway normally stimulated by the β1 adrenergic

receptor at cardiac level (Hidalgo & Granzier, 2013). In addition, in hypertrophied cardiomyocytes from rats treated with the β1-adrenergic receptor agonist isoproterenol, an increased residual actin-myosin cross bridge formation was observed, an additional factor that decreases myocardial relaxation (Sumita Yoshikawa et al., 2013). These results point towards a link between cardiomyocyte hypertrophy and diastolic dysfunction, yet, the relative importance of ventricular hypertrophy on the development of diastolic dysfunction needs further research (Wan et al., 2014).

Another factor mediating diastolic dysfunction is altered cardiomyocyte relaxation, an active process modulated by myocardial and systemic factors (Silverthorn, 2013). Cardiomyocyte relaxation is related to alterations in intracellular calcium handling (Slama et al., 2002; Wan et al., 2014) and nitric oxide signalling. A decrease in relaxation, related to a slower decrease in cytosolic calcium content is observed in cardiomyocytes isolated from renovascular hypertensive rats (Yelamarty et al., 1992). Additionally, diastolic dysfunction in SHR is associated with an increase in baseline intracellular calcium in isolated cardiomyocytes and a decrease in the expression of the Sarco(endo)plasmic $Ca^{2+}$-ATPase isoform 2a (SERCA 2a), an enzyme linked to calcium reuptake by the sarcoplasmic reticulum (Dupont et al., 2012; Eisner, Bode, Venetucci, & Trafford, 2013).

Studies in unilaterally nephrectomized, deoxycorticosterone acetate (DOCA)-salt hypertensive mice have shown that diastolic dysfunction is induced by oxidative stress mediated by the uncoupled activity of neural and endothelial nitric oxide synthases (NOS), increasing the production of superoxide ($\cdot O_2-$), and non-selective blocking of NOS with L-NAME reduces superoxide production and reverses diastolic dysfunction (Silberman et al., 2010). The mechanisms of this effect include reduction of the activity of phospholamban, a protein related to the control of SERCA activity. Thus, coupling of nitric oxide synthase, increasing NO production instead of free oxygen radicals, improves relaxation in this model (Silberman et al., 2010). Noteworthy, NO via its downstream mediator cyclic guanosine monophosphate (cGMP) reduces myofilamentary Ca2+ sensitivity and thereby facilitates cross-bridge detachment (Layland, Li, & Shah, 2002).

Alterations in cardiomyocyte calcium handling and subsequent cardiac dysfunction in SHR may be influenced by increased cardiac inflammatory mediators. Injection of IL-1β impairs

myocardial relaxation in mice (Van Tassell, Toldo, Mezzaroma, & Abbate, 2013). Blocking IL-1β with the recombinant IL-1 receptor antagonist Anakinra improves exercise tolerance in patients with heart failure with preserved systolic function (Van Tassell et al., 2014), a clinical syndrome of exercise intolerance due to impaired myocardial relaxation and/or increased stiffness (Borlaug, 2014). These studies suggest an improvement in cardiac function following IL-1β inactivation (Van Tassell et al., 2014). Also, in SHR, 16 weeks of moderate treadmill exercise improves diastolic function in association with a decrease in myocardial TNF-α and IL-1β and attenuated activity of nuclear factor κB (NF-κB) (Agarwal et al., 2009), a transcription factor that, among other functions, controls the production of pro-inflammatory cytokines as well as enzymes that increase oxidative stress in cardiac cells, including inducible nitric oxide synthase (iNOS) (Elks et al., 2009; Sun et al., 2009).

Accordingly, pro-inflammatory cytokines may activate nitric oxide synthase (NOS) via NF-κB, which, in turn, increases oxygen free radicals, decreases SERCA activity, increases resting intracellular calcium levels, and reduces diastolic function.

In this respect, our studies in SHR show that 4-week-treatment with moxonidine decreases cardiac levels of IL-1β and TNF-α, probably reducing ventricular oxidative stress, and improving calcium handling, an effect likely to improve the active phase of diastole. This mechanism together with the decrease in collagen deposition, may contribute to the observed improvement in diastolic function following activation of imidazoline $I_1$-receptors (Aceros et al., 2011).

The progressive decline in left ventricular function involves apoptotic cardiomyocyte loss. Apoptosis can be induced via the extrinsic (for example through the TNF receptor) or, more importantly in heart failure, the intrinsic pathway (Konstantinidis, Whelan, & Kitsis, 2012; Orogo & Gustafsson, 2013). The intrinsic pathway is regulated by the balance in the activity of the mitochondrial pro-apoptotic protein, Bax and the anti-apoptotic protein, Bcl-2, a balance that maintains mitochondrial activity (Konstantinidis et al., 2012; Orogo & Gustafsson, 2013). The increase in Bax in cardiomyocytes is induced by inflammatory iNOS, and the subsequent production of the NO degradation product peroxynitrite (ONOO-) (Arstall, Sawyer, Fukazawa, & Kelly, 1999). On the other hand, ischemia-induced apoptosis is inhibited by the phosphodiesterase-5 inhibitor sildenafil, by mechanisms that included an

iNOS-dependent increase in Bcl-2 (Das, Xi, & Kukreja, 2005). Together, these studies reveal a dual NO effect: a direct NO-Bcl-2-mediated anti-apoptotic and an indirect NO-peroxynitrite-Bax apoptotic effects.

Moxonidine results in a transient apoptotic effect in SHR ventricles, increasing it at the onset of treatment, but decreasing it by the end of a 4-week-treatment (Paquette et al., 2008). Based on the improved cardiac function, the early apoptotic response most likely involves susceptible (myo)fibroblasts, while cardiomyocytes remain protected. Similar anti-inflammatory and protective effects have been shown in cardiomyopathic hamsters, where 4 week-treatment with moxonidine reduces ventricular cell death, an effect associated with increased Bcl-2 expression (Stabile et al., 2011), suggesting a decrease in peroxynitrite production and a local anti-oxidative effect.

Thus, decreased oxidative stress mediated by inhibition of SNS activity, changes in inflammatory mediators, and decreased nitric oxide synthase activity, may also explain the observed improvement in cardiac function.

The BIO14.6 cardiomyopathic hamster has been described as a model of necrosis-induced heart failure (Gertz, 1972; Kyoi et al., 2006; Strobeck et al., 1979). Our studies in BIO14.6 hamster hearts revealed elevated number of TUNEL positive cardiomyocytes. The number of these cells decreased following moxonidine treatment, in association with a decrease in the ventricular Bax/Bcl-2 ratio (Stabile et al., 2011). Noteworthy, TUNEL assay is commonly used to identify apoptotic cells in situ. However because this assay detects double-and single-stranded DNA fragmentation that is also produced by necrotic DNA degradation in different cell types, including cardiomyocytes (Ohno et al., 1998; Widlak, Li, Wang, & Garrard, 2000), cell death in the myopathic hamster heart may be caused by both necrotic and apoptotic mechanisms. On the other hand, the Bax to Bcl-2 ratio has been related to cardiomyocyte apoptosis, Bax, being pro-apoptotic and Bcl-2 anti-apoptotic (Elsasser, Suzuki, & Schaper, 2000; Hofstaetter, Taimor, Inserte, Garcia-Dorado, & Piper, 2002). In addition, our present in vitro studies reveal that moxonidine opposes starvation-induced cardiomyocyte apoptosis measured by FACS (Aceros, Farah, Noiseux, & Mukaddam-Daher, 2014). Accordingly, cardiac cell protection by moxonidine includes, at least in part, an anti-apoptotic mechanism.

These studies reveal that moxonidine does not deteriorate, rather, improves myocardial function, possibly by imidazoline $I_1$-receptor mediated anti-inflammatory and anti-oxidative mechanisms that hinder cardiac hypertrophy, cardiomyocyte death, and fibrosis. The proposed mechanisms for moxonidine actions are presented in Figure 16.

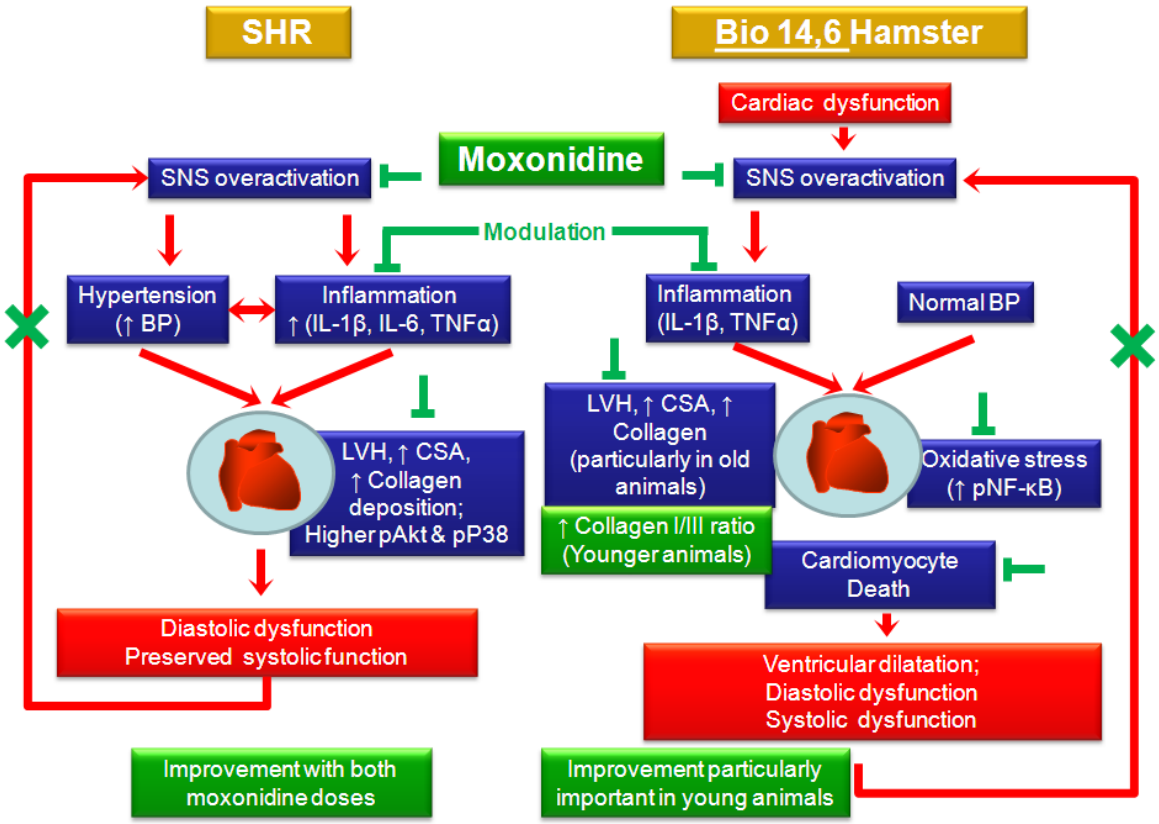



Figure 16: Cardioprotective effects of in vivo moxonidine treatment in hypertensive SHR and Bio14.6 hamsters:

Increased SNS activity and subsequent release of neurotransmitter norepinephrine (NE) is implicated in the pathogenesis and maintenance of hypertension in SHR. SNS overactivity increases the secretion of systemic (IL-1β and IL-6) and cardiac (IL-1β) proinflammatory cytokines. Neurohormones and cytokines lead to morphological changes (LVH and fibrosis, manifested by increased cardiomyocyte surface area (CSA) and collagen deposition) and to functional changes (diastolic dysfunction, shown by increased IVRTc, increased E deceleration rate and decreased E deceleration time, and decreased general myocardial performance, shown as increased LV performance index (LVMPI)), together with preserved systolic function (normal percent ejection fraction (%EF)). Eventually, these changes lead to heart failure (reduced both, diastolic and systolic cardiac functions). In vivo moxonidine (in green) improves diastolic function and global LV performance (decreased LVMPI) and results in LVH regression (reduced cardiomyocyte CSA and fibrosis). These effects are associated

with modulated inflammatory responses (decreased circulating IL-6 and TNFα, as well as decreased cardiac IL-1β) and reduced high Akt and p38 phosphorylation.

On the other hand, the Bio14.6 hamster is a genetic model of heart failure. It is characterized by age-dependent progressive cardiac hypertrophy followed by dilatation and cardiac dysfunction which result in neurohormonal activation and inflammation, causing ventricular cell death and fibrosis, thus leading to further ventricular diastolic and systolic functions. Treatment with moxonidine prevents/delays systolic and diastolic cardiac dysfunctions and ventricular dilatation (increased collagen I/III ratio and reduced cardiomyocyte apoptosis) and modulates inflammatory responses (increased IL-1β and decreased circulating IL-6 and cardiac IL-1β and TNFα and inflammatory markers, pNF-kB and iNOS).

The benefits of in vivo moxonidine may be secondary to inhibition of SNS overactivity, which may improve cardiac function and reduce circulating neurohormones and cytokines levels and their downstream death and fibrotic actions, in association with blood pressure reduction (high concentrations in SHR) or no effect (low concentrations in SHR, low and high concentrations in BIO14.6). In addition, moxonidine, at least in part, may act directly on the heart to interfere with the actions of NE and cytokines. This hypothesis emerges from the pressure-independent cardioprotective effects of moxonidine as well as on the presence of adrenergic and cytokine receptors and imidazoline I1-receptors on cardiac cells.

Most of moxonidine's benefits were observed in response to high, hypotensive concentrations, typically mediated by central imidazoline $I_1$-receptor activation and subsequent inhibition of SNS activity (El-Mas et al., 2009; Zhang & Abdel-Rahman, 2008). However, these benefits also occurred in response to low, sub-hypotensive concentrations, pointing to a local action, mediated by cardiac imidazoline $I_1$-receptors. The participation of cardiac imidazoline $I_1$-receptors in LVH regression, and the mechanisms involved in whole animal studies may be masked by the antihypertensive and sympatholytic effects of the drug. Therefore, in order to selectively investigate cardiac actions, the interaction of moxonidine with oxidative and inflammatory markers was investigated *in vitro* on primary cardiac cells in culture.

## 4.2 *In vitro* imidazoline $I_1$-receptor activation by moxonidine protects cardiomyocytes against inflammatory and oxidative stressors. Opposite effects occur in fibroblasts

As regression of LVH by moxonidine may include cardiac cell hypertrophy, proliferation, and death, the direct effect of moxonidine on hypertension and heart failure-induced cardiac cell growth and death and the implicated signaling proteins were investigated *in vitro*, on primary cardiomyocytes and fibroblasts in culture, in the absence of confounding mechanisms.

Neonatal but not adult rat cardiac cells have been chosen because they are easier to handle, and can be grown and maintained *in vitro* longer times. More importantly, although the use of neonatal cell data may not be translated to normal adult cells, they may be translated to pathological situation, such as the hypertrophied heart, in which the fetal gene program is re-initiated (Ames, Lawson, Mackey, & Holmes, 2013). As for neonatal rat cardiac fibroblasts, these cells have been extensively used to identify potential pro-fibrotic factors, and elucidate the signalling pathways coupled to growth and ECM protein expression (Peng, Dai, Ji, & Dai, 2010).

Using primary cultures of neonatal rat cardiac myocytes and fibroblasts, the present studies identify cardiac cell type-specific signalling pathways of imidazoline $I_1$-receptors/nischarin. They also demonstrate that moxonidine protects cardiomyocytes and modulates cardiac fibroblast survival in the presence of oxidative and inflammatory stimuli, namely norepinephrine, TNF-α and IL-1β. These studies reveal that activation of imidazoline $I_1$-receptors by moxonidine does not only inhibit the production of norepinephrine and cytokines but also directly interferes with their actions in cardiac cells (Aceros et al., 2011; Aceros et al., 2014).

Norepinephrine induces cardiomyocyte death by increasing oxidative stress and downstream activation of p38 MAPK, followed by secretion of TNF-α. Blocking any of these mediators reduces norepinephrine-induced cardiomyocyte death (Chen, Jiang, Wan, Bi, & Yuan, 2012; Fu et al., 2004; Iwai-Kanai et al., 2001). In addition, IL-1β increases cardiomyocyte mortality

by mechanisms that include the activation of p38 MAPK and iNOS (Arstall et al., 1999; Shindo et al., 1995).

The effects of iNOS are mediated by its nitric oxide (NO) production. However, as mentioned above, NO exerts both pro- and anti-apoptotic effects in cardiomyocytes, depending on quantities and downstream signalling pathways. Low NO concentrations are anti-apoptotic, increasing Bcl-2 expression and inhibiting caspase-3 by S-nitrosylation. On the other hand, high NO concentrations induce apoptosis by increasing Bax and activating the death receptor (reviewed by Ravazi et al., 2005) (Razavi, Hamilton, & Feng, 2005), as well as formation of the free radical peroxynitrite (ONOO-). Peroxynitrite, which triggers both apoptosis and necrosis (Levrand et al., 2006), is an NO degradation product generated by combination of NO with oxidants. Through activation of the pro-survival/anti-apoptotic signals, as well as through its antioxidant effect, moxonidine may shift the balance towards the anti-apoptotic effect of NO.

The present studies show that co-incubation with moxonidine reduces norepinephrine- and IL-1β-induced cardiomyocyte mortality, mainly by decreasing p38 MAPK activation via the imidazoline $I_1$-receptor (Aceros et al., 2011), and by shifting the p38 MAPK-iNOS pathway from stimulation to inhibition of cell death (Aceros et al., 2014). An anti-oxidative action of imidazoline $I_1$-receptor activation by moxonidine may underlie these actions. The anti-oxidative effect of imidazoline $I_1$-receptor/nischarin has been demonstrated in HEK293 cells, where nischarin-transfection improved cell survival (Aceros, Cobos-Puc, Farah, Noiseux, & Mukaddam-Daher, 2013), and in cardiomyocytes where moxonidine opposed $H_2O_2$-induced cell death (Aceros et al., 2014). It is also consistent with the recent *in vivo* finding that moxonidine decreases nitrite/nitrate production and increases the anti-oxidative enzymes, superoxide dismutase and catalase, in brains of rats challenged with the mitochondrial complex II inhibitor 3-Nitropropionic acid, a model of Huntington's disease (Gupta & Sharma, 2014).

In addition to an anti-oxidative effect, moxonidine, through imidazoline $I_1$-receptors, may activate the p38 MAPK and Akt/mammalian target of rapamycin (mTOR) cardioprotective pathway, while inhibiting the p38 MAPK-mediated death pathway. This hypothesis is supported by the protective effects of parallel activation of p38 MAPK and Akt, followed by

activation of mTOR observed in neonatal rat cardiomyocytes exposed to $H_2O_2$ (Hernandez et al., 2011), as well as with the finding that the protective mTOR pathway in cardiomyocytes is activated by the IRS1/2-Akt pathway (Troncoso et al., 2013). Imidazoline $I_1$-receptor signalling pathway includes IRS1-4/Akt (Edwards et al., 2012; Sano et al., 2002). Noteworthy, IRS proteins are the main targets for the insulin-activated protein tyrosine kinase. After phosphorylation, IRSs associate with the enzyme PI3-kinase, which upon activation, leads to translocation of the glucose transporter, Glut-4. Under basal conditions, Glut-4 protein is stored in intracellular vesicles, and upon stimulation, is translocated to the plasma membrane, increasing the membrane capacity for glucose transport (Chang, Chiang, & Saltiel, 2004). Increased glucose uptake reduces hypoxia-induced apoptosis in cultured neonatal rat cardiomyocytes (Malhotra & Brosius, 1999). Importantly, the imidazoline $I_1$-receptor signalling pathway includes IRS1-4/Akt (Edwards et al., 2012; Sano et al., 2002), supporting the possibility that moxonidine could activate the protective mTOR pathway via imidazoline $I_1$-receptors.

In short, imidazoline $I_1$-receptor-mediated anti-oxidative mechanism, with parallel activation of the pro-survival IRS/Akt pathway may explain the observed *in vitro* local moxonidine-imidazoline $I_1$-receptor-mediated cardiomyocyte protection.

On the other hand, the observed effects of moxonidine in fibroblasts are mostly opposite to those observed in cardiomyocytes. The imidazoline $I_1$-receptor/moxonidine mediated protective anti-oxidative mechanism observed in cardiomyocytes is absent in fibroblasts. Also, in contrast to cardiomyocytes, moxonidine stimulates basal and IL-1β-induced fibroblast mortality by inhibition of JNK and iNOS (Aceros et al., 2011; Aceros et al., 2014). Cell-type selective effects have been previously shown in response to endothelin. Endothelin induces cardiomyocyte hypertrophy and fibroblast proliferation (Drimal, Mislovicova, Ismail, & Moncek, 1999; Gan, Chakrabarti, & Karmazyn, 2003; You et al., 2006), through local activation of endothelin A receptor, a G-protein coupled receptor that classically binds to a $G\alpha_{q/11}$ protein, activating phospholipase C, and increasing inositol phosphate production (Ivey, Osman, & Little, 2008). However, endothelin A receptor activation markedly increases inositol phosphate production in cardiomyocytes but not in fibroblasts (Hilal-Dandan, Urasawa, & Brunton, 1992; Meszaros et al., 2000). These studies show that the same receptor

has cell-selective alternative signalling pathways. Similar observations have been reported for the angiotensin 1 receptor and the prostaglandin E2 receptor (Meszaros et al., 2000), a situation that may also apply for the imidazoline-$I_1$ receptor.

The differential actions of moxonidine on cardiomyocytes and fibroblasts, especially on cell survival and iNOS induction in the presence of stressors may depend on several factors, including a differential effect on ANP secretion. Moxonidine stimulates the release of ANP from neonatal cardiomyocytes following imidazoline $I_1$-receptor activation *in vitro* (Figure 14, Supplementary data). Noteworthy, the presence of ANP in the culture medium potentiates IL-1β-induced iNOS expression in cardiomyocytes (Horio et al., 1998; Yamamoto et al., 1997). This mechanism is not observed in fibroblasts, where ANP release is absent or negligible.

In addition, cardiomyocytes and fibroblasts express different subtypes of sphingosine 1 phosphate (S1P) receptors: cardiomyocytes mainly express S1P1 and fibroblasts mainly express S1P3 receptors (Means & Brown, 2009). Molderings et al. (Molderings et al., 2007) have shown that imidazoline $I_1$-receptor agonists, including moxonidine, interact with both S1P1 and S1P3. S1P inhibits IL-1β induction of NO production and iNOS expression in rat vascular smooth muscle cells (Machida et al., 2008), a proliferative cell type closely related to cardiac fibroblasts in culture (myofibroblasts) and, like fibroblasts, expresses S1P3 receptors. The effect of S1P is similar to the observed decrease in iNOS following co-incubation of fibroblasts with Il-1β and moxonidine. It is not known whether an opposite effect through S1P1 receptors occurs in cardiomyocytes, however, S1P protects cardiomyocytes from hypoxia-induced cell death (Karliner, Honbo, Summers, Gray, & Goetzl, 2001).

On the other hand, it has been shown that the imidazoline $I_1$-receptor/nischarin with the α5 integrin subunit (Alahari et al., 2000). In normal cardiomyocytes α5 integrin expression is scarce; while is normally expressed in fibroblasts. The expression of the α5 integrin subunit is differentially regulated in ventricular hypertrophy, decreasing in fibroblasts and increasing in cardiomyocytes (Burgess et al., 2002; Ross, 2002). The expression of the α5 integrin subunit may influence the imidazoline $I_1$-receptor/nischarin sub-cellular localization, possibly by anchoring it to the membrane. The imidazoline $I_1$-receptor/nischarin is a cytosolic protein, as we have demonstrated by overexpressing it in HEK293 cells (Aceros et al., 2013), and those in Cos7, Neuro 2A and 293T cells (Alahari et al., 2000; Jain et al., 2013), as well as a

membrane-associated protein in PC12 cells (Zhang & Abdel-Rahman, 2006). Interestingly, this difference in localization is related to a functional difference. In HEK293 (Aceros et al., 2013) and 293T cells expressing nischarin, an anti-proliferative effect was observed when compared to non-expressing cells (Jain et al., 2013), suggesting that the sub-cellular localization may influence imidazoline $I_1$-receptor/nischarin effects. Accordingly, differential α5 integrin subunit expression in cardiomyocytes and fibroblasts might influence nischarin's localization and subsequently, the mechanisms that differentiate cellular responses to moxonidine.

Thus, differential receptor expression and/or subsequent availability of different signalling pathways as well as the activation of distinct downstream targets in cardiomyocytes and fibroblasts may underlie the observed different cell-type selective physiological effects.

Finally, an important finding that emerges from our previous and present work is imidazoline $I_1$-receptor regulation in response to endogenous and exogenous factors. *Ex vivo* perfusion of isolated normotensive rat hearts with imidazoline compounds stimulates ANP release (Mukaddam-Daher et al., 1997; Mukaddam-Daher et al., 2006), with greater effect observed in SHR hearts (Figure 15, Supplementary data). These studies demonstrate that cardiac imidazoline $I_1$-receptors are functional without the contribution of the central nervous system and suggest a possible up-regulation in hypertensive hearts. Binding studies have confirmed that heart imidazoline $I_1$-receptors are up-regulated in rat hypertension and hamster and human heart failure and have shown that heart imidazoline $I_1$-receptors are normalized in SHR by chronic *in vivo* treatment with moxonidine (El-Ayoubi et al., 2002; El-Ayoubi et al., 2003, 2004). In contrast, heart imidazoline $I_1$-receptors/nischarin are further up-regulated by in vivo moxonidine in hamster heart failure (Stabile et al., 2011). In vitro studies extend to identify imidazoline $I_1$-receptor/nischarin mRNA and protein in both cardiac cell types, fibroblasts and myocytes, and show that imidazoline $I_1$-receptor/nischarin protein is targeted by treatment, thus regulating its expression, in a cell-selective and stimulus-specific manner (Aceros et al., 2014).

Recent studies by Alahari's group show higher expression of nischarin mRNA in normal human breast tissue than tumor tissue, associated with decreased α5 integrin expression, FAK phosphorylation, and Rac activation (Baranwal et al., 2011). They also show that nischarin

suppression leads to faster cell growth, consistent with our results showing anti-proliferative action of nischarin in fibroblasts. Therefore, based on the link of nischarin to integrin signaling and on its intrinsic antiproliferative action mediated by Rac1 inhibition, together with our studies showing up-regulated nischarin in myopathic hearts, it is tempting to speculate that nischarin up-regulation is an adaptive response of the myocardium to pressure/volume overload and point to cardiac nischarin overexpression as a protective mechanism, contributing to LVH control through an anti-oxidative action. Alternatively, cardiac nischarin suppression in the presence of LVH may be deleterious.

## 4.3 Conclusion

This work on various experimental models mimicking human cardiomyopathies, shows that activation of imidazoline $I_1$-receptors protects hypertensive hearts from LVH and slows the functional decay in heart failure. Importantly, moxonidine does not deteriorate cardiac function; it rather improves it and reduces mortality in heart failure hamsters, suggesting that the negative effects observed in clinical trials may not be related to a direct deleterious effect of the imidazoline $I_1$-receptor on cardiac function.

Furthermore, a unifying protective mechanism is suggested by this work. The *in vivo* and *in vitro* experiments propound that the presence and/or activation of the imidazoline $I_1$-receptors exert anti-inflammatory, and anti-oxidative effects, which appear to be particularly important in cardiomyocytes, reducing the formation of peroxynitrite, thus probably switching excess NO actions from deleterious to protective. This effect is not observed in cardiac fibroblasts. In fact, activation of imidazoline $I_1$-receptors induces fibroblast death *in vitro.* Since abnormal fibroblast proliferation is observed in LVH (Cacciapuoti, 2011), and fibroblasts are the main producer of extracellular matrix and collagen in cardiac tissue (Jugdutt & Amy, 1986; Kuwahara et al., 2003; Weber et al., 1992), imidazoline $I_1$-receptor mediated fibroblast death may mediate the reduced fibrosis *in vivo*, together with improvement of cardiac structure and function following pharmacological treatment (Aceros et al., 2011; Aceros et al., 2014; Stabile et al., 2011).

These results support the hypothesis that local activity of imidazoline $I_1$-receptors protects cardiomyocytes under stress.

## 4.4 Perspectives

Cell culture studies have revealed that activation of nischarin results in protective effects in cardiomyocytes and anti-proliferative actions in fibroblasts (Aceros et al., 2014). In an attempt to further explore nischarin's signalling pathways, specifically those involved in the modulation of action of NO, lipid-mediated transfection (Lipofectamine 2000, Invitrogen, Burlington, ON) with GFP tagged-nischarin cDNA was performed in both cardiomyocytes and fibroblasts, similarly to that performed on HEK293 cells (Aceros et al., 2013). Efficiency of this approach was low, with transfections efficiencies of around 10% after optimization. This efficiency is similar to the one reported in the literature for this technique (Louch, Sheehan, & Wolska, 2011), yet, is too low to allow for clear evaluation of signalling and survival differences between GFP-nischarin transfected and GFP-vector transfected cardiomyocytes. However, better transduction can be achieved by viral vector, especially in cardiac cells and when the protein is as big (~250 kDa). Figure 17 includes preliminary *in vitro* lentiviral-GFP overexpression in rat cardiomyocytes and fibroblasts, demonstrating feasibility of the proposed experimental approach. Transfection with lentivirus resulted in 41% of cardiomyocytes and 62% of fibroblasts as GFP positive, 24h after viral infection, an efficiency that would be sufficient to give meaningful results.

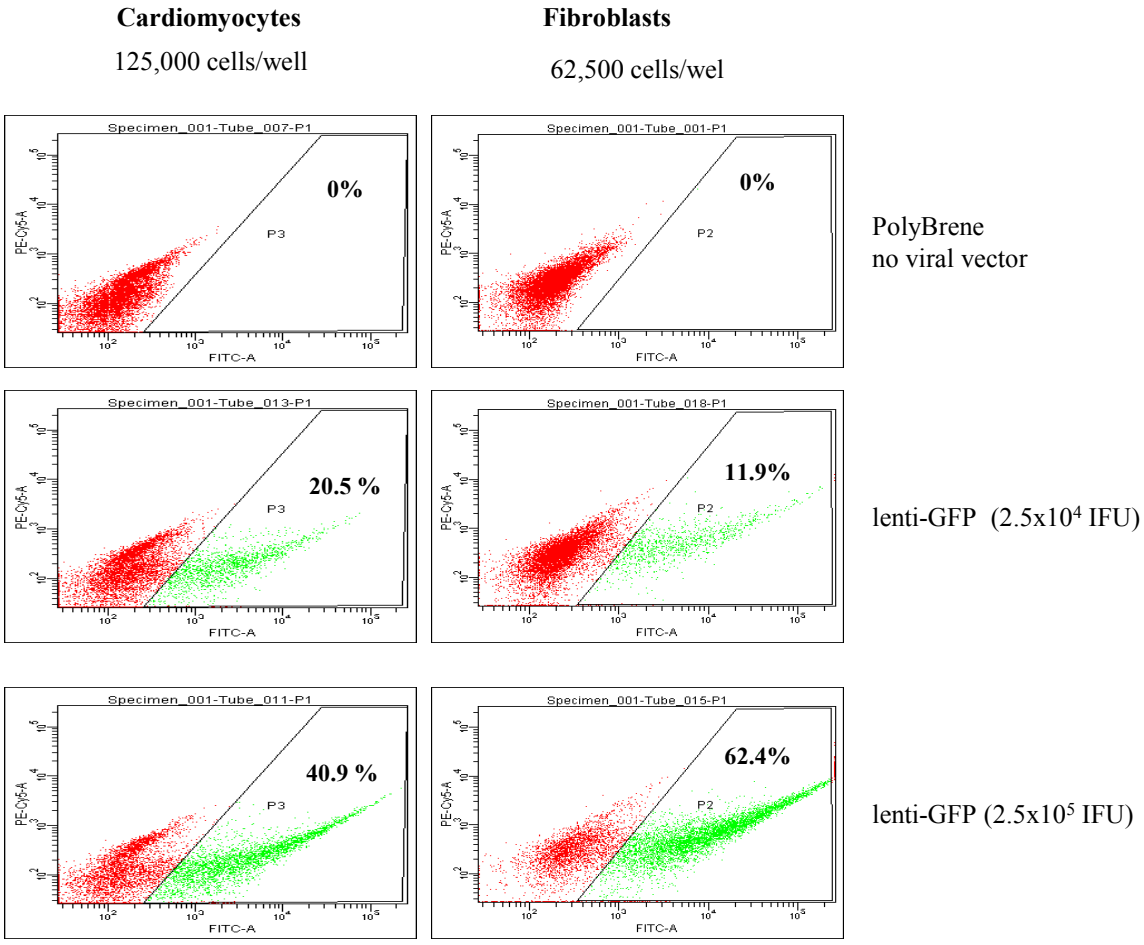



Figure 17: Flow cytometry images showing the percentage of GFP positive cardiomyocytes or fibroblasts after lentiviral infection ($2.5x10^4$ infectious units (IFU) and $2.5x10^5$ IFU).

This method would lead to better evaluation of nischarin signalling and functions in cardiac cells. Once viral transfection of cardiomyocytes and fibroblasts with GFP-tagged-nischarin cDNA or GFP-empty vector as control is performed, cells will be incubated with NE and IL-1β, stimuli that have induced differential effects on survival, whether alone or in the presence of moxonidine. Cell survival and signalling pathways involved will be investigated by the use of specific inhibitors. Also, since the proposed protective mechanism of moxonidine includes an antioxidant effect, it would be important to measure the activity levels of antioxidant enzymes like superoxide dismutase and catalase (Gupta & Sharma, 2014).

In view of altered nischarin levels in cardiovascular diseases and in response to treatment, studies are required to determine the impact of *in vivo* cardiac nischarin modulation on cardiac

structural, functional, and metabolic parameters, in basal conditions and in response to a chronic hemodynamic stress, such as that induced by aortic banding, or in ischemia-induced heart failure, such as that induced by coronary artery ligation (Houser et al., 2012). The latter is based on Grassi's (Grassi, 2013) proposal that activation of imidazoline $I_1$-receptors might not be as effective in this type of heart failure because of the deleterious effects observed in ischemic heart failure patients following moxonidine treatment (Cohn et al., 2003).

Furthermore, based on the improved metabolic profile by moxonidine in patients with metabolic syndrome and diabetes, and on our studies revealing improved cardiac function independently of blood pressure control, the use of the diabetic cardiomyopathy model is also warranted. Diabetic cardiomyopathy depends on hyperglycemia-induced increase in cardiac oxidative stress, followed by increase ROS production and inflammation, leading to fibrosis and cardiac dysfunction (Boudina & Abel, 2007; Pappachan et al., 2013; Schilling & Mann, 2012; Varga et al., 2014). In streptozotocin-treated rats (a model of type 1 diabetes), treatment with the non-selective α2 adrenergic/imidazoline $I_1$-receptor agonist clonidine in hypotensive doses abolished the diabetes-induced decrease in systolic function (Satia, Damani, & Goyal, 1997), suggesting a beneficial effect of imidazoline $I_1$-receptor activation in cardiac function.

We expect that nischarin over-expression *in vivo* will attenuate pressure overload- or metabolic-induced hypertrophy and fibrosis, and preserve left ventricular function, together with the prevention of enhanced ROS formation. Upregulation of imidazoline $I_1$-receptor/nischarin in the heart will not only improve diastolic dysfunction (as observed in the cardiac sympathetic denervation model (Wang, Wang, Cornish, Rozanski, & Zucker, 2014)), but would also prevent or delay some of the long term systolic dysfunction, demonstrating that cardiac nischarin levels may account for cardiac protection. Studies will also show that cardiac specific nischarin up-regulation potentiates the cardiac response to moxonidine. We speculate that nischarin suppression will result in opposite effects. Both ways, they will demonstrate that nischarin expression level/distribution could account for many aspects of cardiac remodelling, emphasizing its potential importance as an anti-hypertrophic and anti-fibrotic target.

In this respect we have planned to modulate nischarin levels in mice and to perform, simultaneous studies on wild type mice, nischarin knock-out mice (nischarin-/-), and knock-out mice with cardiac-specific expression of nischarin (c-nischarin). We considered that the

nischarin knock-out mouse model (nischarin-/-) would offer new information about the importance of nischarin in cardiovascular control. It would also resolve the everlasting debate concerning the question whether imidazoline compounds mediate their effects through imidazoline receptors (nischarin) or α2-adrenergic receptors. This debate is based on the lack of specific agonists and antagonists as well as on studies showing that the antihypertensive effect of imidazoline compounds is absent in animals with mutation in the α2-adrenergic receptor (Zhu et al., 1999). Also, in addition to cardiovascular research, generating this model could be an important achievement for diabetes and cancer research.

We have already started creating nischarin knock-out model. We received from TGIM embryonic stem cells with mutations of nischarin. Clones D6 and H7 were injected in mouse embryos and successfully gave 2 male and 1 female chimeras (Figure 18).

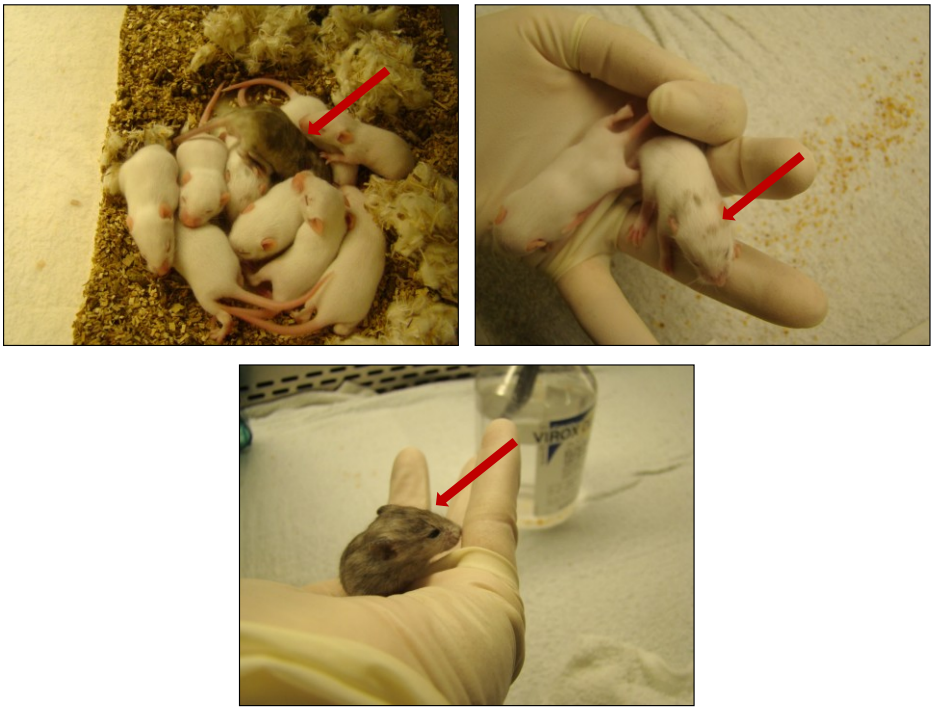


Figure 18: Three different clones of nischarin knockout embryonic stem cells, originated in C57BL/6 mice, were laser injected in to CD-1 8-cell to non-compacted morula stage albino mouse embryos to generate Nischarin chimeras. The embryos were cultured overnight to the blastocyst stage of embryonic development, and then transferred into pseudo-pregnant females that had been mated to vasectomised males. Pups were born 16-19 days after surgery and chimeras were identified by coat color. In the images the change in coat color (arrows) characteristic of chimeric animals is observed and contrasted with white-coated littermates.

After mating the chimeras with normal C57BL/6 animals, and selection of dark coat offspring, polymerase chain reaction (PCR) of genomic DNA extracted from tail snips was performed using primers for complete and KO nischarin sequences, giving 2 separate base pairs levels in cases of heterozygosis (Figure 19).

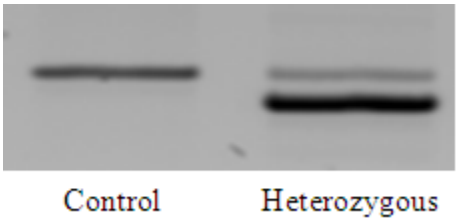



Figure 19: PCR gel image of normal and heterozygous nischarin genomic DNA samples derived from tail snips.

Unfortunately, the mating did not result in viable heterozygous animals. The only confirmed heterozygous mouse born (offspring of one of the male chimeras, confirmed by the PCR showed in figure 19) died shortly after birth, and several cycles of mating over the course of 1 year did not provide any new heterozygous animals. Sacrifice of pregnant animals, searching from embryologic evidence of *in utero* KO at different gestation times did not show any evidence of KO animals being produced, thus suggesting that nischarin KO was lethal pre-implantation or that the chimeric spermatozoids were less fit (in quantity, quality or both) to fertilize ova.

The failure of this experimental approach does not reduce the interest of observing the cardiac results of modulation of the imidazoline $I_1$-receptor, so it would be appropriate to develop a local cardiac nischarin expression and KO, an approach that would likely have better survival.

Tissue specific nischarin KO can be achieved by the creation of a Cre-loxP based cardiac specific KO mouse (Moga, Nakamura, & Robbins, 2008), using commercially available nischarin-loxP embryonic stem cells, to develop homozygous nischarin-loxP mouse, animals that will maintain normal nischarin expression, then mate them with mouse expressing the Cre recombinase enzyme, driven by the cardiomyocyte-specific promoter α-myosin heavy chain, thus expressing Cre only in adult cardiomyocytes. Since the expression of Cre will delete the

nischarin-loxP gene in the tissues were it is expressed (Doetschman & Azhar, 2012; Moga et al., 2008), the offspring of these animals will have a KO of nischarin in their cardiomyocytes.

Nischarin overexpression can be achieved under the control of the α-myosin heavy chain promoter (c-nischarin), by creating a plasmid in which full length sequence of nischarin is ligated to the cardiac-specific murine alpha-myosin heavy chain promoter, then this plasmid is injected in embryonic stem cells, which are later implanted in pseudo-pregnant females. Cross breed of the offspring will allow the development of homozygous c-nischarin mice (Yarbrough et al., 2014).

Alternatively, nischarin overexpression in the heart can be achieved by means of a lentiviral vector containing genomic nischarin DNA and enhanced green fluorescent protein (EGFP) controlled by cytomegalovirus (Metcalfe et al., 2004). The vector will be injected in the ventricular cavity of 5-day-old neonatal mice (or rats). At this stage, cardiac cells are still dividing and immune system not yet developed. This approach has been frequently used (Bonci et al., 2003; Metcalfe et al., 2004; Yoshimitsu et al., 2006). The hearts of neonatal mice/rats are visible under the transparent skin and injection into the left ventricular cavity would only require a single injection and small volume. Pups recover within minutes after the injection, and almost 100% of them survive the process. The use of virus-based vectors for gene transfer in the heart has emerged as a powerful approach to over-express and access the function of key enzymes and proteins in healthy or diseased hearts. High-efficiency infection of the heart *in vivo* has been achieved in mice and rats (Champion et al., 2003; Donahue, Kikkawa, Johns, Marban, & Lawrence, 1997; Hart et al., 2008). The lentiviral vector-mediated gene delivery system has several advantages over other viral or non-viral gene delivery systems: high efficiency of gene transfer into a wide variety of cells including both dividing and non-dividing cells, long-term infection due to gene integration into the chromosome of host cells, and no toxicity or immune response (Abbas-Terki, Blanco-Bose, Deglon, Pralong, & Aebischer, 2002; Hart et al., 2008).

Using a similar approach, endogenous nischarin will be inhibited by intra-cardiac injection of the same lentiviral vector, but harbouring a 21 base pair siRNA oligonucleotide (ODN) (or corresponding mismatched ODN) directed against rat/mouse nischarin. This siRNA has been

shown to be effective in PC12 cells and NIH3T3 cells *in vitro*, and in the brain *in vivo* (Zhang & Abdel-Rahman, 2006, 2008).

These studies will demonstrate that nischarin expression level/distribution could account for many aspects of cardiac remodelling, emphasizing its potential importance as an anti-hypertrophic and anti-fibrotic target. The results, if positive, will push towards the development of specific imidazoline $I_1$-receptor agonists that do not cross the blood-brain barrier, and thus are devoid of the central hypotensive effects (Chan et al., 2005; Haxhiu et al., 1994). While the antihypertensive action of centrally acting imidazoline compounds is appreciated, development of new cardiac-selective $I_1$-receptor agonists may confer additional benefits with less systemic adverse effects. In addition, specifically-tailored cell/gene-driven nischarin treatments could eventually be important for patients with heart disease.

# Bibliography


Abbas-Terki, T., Blanco-Bose, W., Deglon, N., Pralong, W., & Aebischer, P. (2002). Lentiviral-mediated RNA interference. *Hum Gene Ther, 13*(18), 2197-2201. doi: 10.1089/104303402320987888

Abbate, A., Scarpa, S., Santini, D., Palleiro, J., Vasaturo, F., Miller, J., . . . Baldi, A. (2006). Myocardial expression of survivin, an apoptosis inhibitor, in aging and heart failure. An experimental study in the spontaneously hypertensive rat. *Int J Cardiol, 111*(3), 371-376. doi: 10.1016/j.ijcard.2005.07.061

Abbate, A., Van Tassell, B. W., Biondi-Zoccai, G., Kontos, M. C., Grizzard, J. D., Spillman, D. W., . . . Vetrovec, G. W. (2013). Effects of interleukin-1 blockade with anakinra on adverse cardiac remodeling and heart failure after acute myocardial infarction [from the Virginia Commonwealth University-Anakinra Remodeling Trial (2) (VCU-ART2) pilot study]. *Am J Cardiol, 111*(10), 1394-1400. doi: 10.1016/j.amjcard.2013.01.287

Aceros, H., Cobos-Puc, L., Farah, G., Noiseux, N., & Mukaddam-Daher, S. (2013). Nischarin Over-Expression Opposes Cell-Death Induced By Oxidative Stress. Dans A. Zarzuelo & R. Jimenez (dir.), *Proceedings of the 6th European Congress of Pharmacology* (p. 35-41). 40128 Bologna: Medimond S R L.

Aceros, H., Farah, G., Cobos-Puc, L., Stabile, A. M., Noiseux, N., & Mukaddam-Daher, S. (2011). Moxonidine improves cardiac structure and performance in SHR through inhibition of cytokines, p38 MAPK and Akt. *Br J Pharmacol, 164*(3), 946-957. doi: 10.1111/j.1476-5381.2011.01355.x

Aceros, H., Farah, G., Noiseux, N., & Mukaddam-Daher, S. (2014). Moxonidine modulates cytokine signalling and effects on cardiac cell viability. *Eur J Pharmacol, 740C*, 168-182. doi: 10.1016/j.ejphar.2014.06.047

Agarwal, D., Haque, M., Sriramula, S., Mariappan, N., Pariaut, R., & Francis, J. (2009). Role of proinflammatory cytokines and redox homeostasis in exercise-induced delayed progression of hypertension in spontaneously hypertensive rats. *Hypertension, 54*(6), 1393-1400. doi: 10.1161/hypertensionaha.109.135459

Aguilera, G. (1992). Role of angiotensin II receptor subtypes on the regulation of aldosterone secretion in the adrenal glomerulosa zone in the rat. *Mol Cell Endocrinol, 90*(1), 53-60.

Ahn, J., & Kim, J. (2012). Mechanisms and consequences of inflammatory signaling in the myocardium. *Curr Hypertens Rep, 14*(6), 510-516. doi: 10.1007/s11906-012-0309-0

Aikawa, R., Nagai, T., Kudoh, S., Zou, Y., Tanaka, M., Tamura, M., . . . Komuro, I. (2002). Integrins play a critical role in mechanical stress-induced p38 MAPK activation. *Hypertension, 39*(2), 233-238.

Alahari, S. K. (2003). Nischarin inhibits Rac induced migration and invasion of epithelial cells by affecting signaling cascades involving PAK. *Exp Cell Res, 288*(2), 415-424.

Alahari, S. K., Lee, J. W., & Juliano, R. L. (2000). Nischarin, a novel protein that interacts with the integrin alpha5 subunit and inhibits cell migration. *J Cell Biol, 151*(6), 1141-1154.

Amann, K., Greber, D., Gharehbaghi, H., Wiest, G., Lange, B., Ganten, U., . . . Mall, G. (1992). Effects of nifedipine and moxonidine on cardiac structure in spontaneously hypertensive rats. Stereological studies on myocytes, capillaries, arteries, and cardiac interstitium. *Am J Hypertens, 5*(2), 76-83.

Ambler, S. K., & Leite, M. F. (1994). Regulation of atrial natriuretic peptide secretion by alpha 1-adrenergic receptors: the role of different second messenger pathways. *J Mol Cell Cardiol, 26*(3), 391-402. doi: 10.1006/jmcc.1994.1048

Ames, E. G., Lawson, M. J., Mackey, A. J., & Holmes, J. W. (2013). Sequencing of mRNA identifies re-expression of fetal splice variants in cardiac hypertrophy. *J Mol Cell Cardiol, 62*, 99-107. doi: 10.1016/j.yjmcc.2013.05.004

Amin, P., Singh, M., & Singh, K. (2011). beta-Adrenergic Receptor-Stimulated Cardiac Myocyte Apoptosis: Role of beta1 Integrins. *J Signal Transduct, 2011*, 179057. doi: 10.1155/2011/179057

Antunes-Rodrigues, J., de Castro, M., Elias, L. L., Valenca, M. M., & McCann, S. M. (2004). Neuroendocrine control of body fluid metabolism. *Physiol Rev, 84*(1), 169-208. doi: 10.1152/physrev.00017.2003

Aranguiz-Urroz, P., Canales, J., Copaja, M., Troncoso, R., Vicencio, J. M., Carrillo, C., . . . Diaz-Araya, G. (2011). Beta(2)-adrenergic receptor regulates cardiac fibroblast autophagy and collagen degradation. *Biochim Biophys Acta, 1812*(1), 23-31. doi: 10.1016/j.bbadis.2010.07.003

Arstall, M. A., Sawyer, D. B., Fukazawa, R., & Kelly, R. A. (1999). Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circ Res, 85*(9), 829-840.

Aukrust, P., Ueland, T., Lien, E., Bendtzen, K., Muller, F., Andreassen, A. K., . . . Gullestad, L. (1999). Cytokine network in congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. *Am J Cardiol, 83*(3), 376-382.

Azevedo, E. R., Newton, G. E., & Parker, J. D. (1999). Cardiac and systemic sympathetic activity in response to clonidine in human heart failure. *J Am Coll Cardiol, 33*(1), 186-191.

Baranwal, S., Wang, Y., Rathinam, R., Lee, J., Jin, L., McGoey, R., . . . Alahari, S. K. (2011). Molecular characterization of the tumor-suppressive function of nischarin in breast cancer. *J Natl Cancer Inst, 103*(20), 1513-1528. doi: 10.1093/jnci/djr350

Baum, J., & Duffy, H. S. (2011). Fibroblasts and myofibroblasts: what are we talking about? *J Cardiovasc Pharmacol, 57*(4), 376-379. doi: 10.1097/FJC.0b013e3182116e39

Becker, D. E. (2012). Basic and clinical pharmacology of autonomic drugs. *Anesth Prog, 59*(4), 159-168; quiz 169. doi: 10.2344/0003-3006-59.4.159

Belenkov, Y., Vikhert, O. A., Belichenko, O. I., & Arabidze, G. G. (1992). Magnetic resonance imaging of cardiac hypertrophy in malignant arterial hypertension. *Am J Hypertens, 5*(6 Pt 2), 195s-199s.

Bernardo, B. C., Weeks, K. L., Pretorius, L., & McMullen, J. R. (2010). Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacol Ther, 128*(1), 191-227. doi: 10.1016/j.pharmthera.2010.04.005

Bettink, S. I., Werner, C., Chen, C. H., Muller, P., Schirmer, S. H., Walenta, K. L., . . . Friedrich, E. B. (2010). Integrin-linked kinase is a central mediator in angiotensin II type 1- and chemokine receptor CXCR4 signaling in myocardial hypertrophy. *Biochem Biophys Res Commun, 397*(2), 208-213. doi: 10.1016/j.bbrc.2010.05.086

Beuschlein, F. (2013). Regulation of aldosterone secretion: from physiology to disease. *Eur J Endocrinol, 168*(6), R85-93. doi: 10.1530/eje-13-0263

Bhambi, B., & Eghbali, M. (1991). Effect of norepinephrine on myocardial collagen gene expression and response of cardiac fibroblasts after norepinephrine treatment. *Am J Pathol, 139*(5), 1131-1142.

Blaine, E. H., & Davis, J. O. (1971). Evidence for a renal vascular mechanism in renin release: new observations with graded stimulation by aortic constriction. *Circ Res, 28*(5), Suppl 2:118-126.

Bogoyevitch, M. A., Andersson, M. B., Gillespie-Brown, J., Clerk, A., Glennon, P. E., Fuller, S. J., & Sugden, P. H. (1996). Adrenergic receptor stimulation of the mitogen-activated protein kinase cascade and cardiac hypertrophy. *Biochem J, 314 ( Pt 1)*, 115-121.

Bohm, M., Swedberg, K., Komajda, M., Borer, J. S., Ford, I., Dubost-Brama, A., . . . Tavazzi, L. (2010). Heart rate as a risk factor in chronic heart failure (SHIFT): the association between heart rate and outcomes in a randomised placebo-controlled trial. *Lancet, 376*(9744), 886-894. doi: 10.1016/s0140-6736(10)61259-7

Bombardini, T. (2005). Myocardial contractility in the echo lab: molecular, cellular and pathophysiological basis. *Cardiovasc Ultrasound, 3*, 27. doi: 10.1186/1476-7120-3-27

Bonci, D., Cittadini, A., Latronico, M. V., Borello, U., Aycock, J. K., Drusco, A., . . . Condorelli, G. (2003). 'Advanced' generation lentiviruses as efficient vectors for cardiomyocyte gene transduction in vitro and in vivo. *Gene Ther, 10*(8), 630-636. doi: 10.1038/sj.gt.3301936

Bonow, R., Mann, D., Zipes, D., Libby, P., & Branwald, E. (2012). *Braunwald's heart disease : a textbook of cardiovascular medicine*. (9e éd.). Elsevier.

Borbely, A., Falcao-Pires, I., van Heerebeek, L., Hamdani, N., Edes, I., Gavina, C., . . . Paulus, W. J. (2009). Hypophosphorylation of the Stiff N2B titin isoform raises cardiomyocyte resting tension in failing human myocardium. *Circ Res, 104*(6), 780-786. doi: 10.1161/circresaha.108.193326

Borbely, A., van der Velden, J., Papp, Z., Bronzwaer, J. G., Edes, I., Stienen, G. J., & Paulus, W. J. (2005). Cardiomyocyte stiffness in diastolic heart failure. *Circulation, 111*(6), 774-781. doi: 10.1161/01.cir.0000155257.33485.6d

Borg, T. K., Johnson, L. D., & Lill, P. H. (1983). Specific attachment of collagen to cardiac myocytes: in vivo and in vitro. *Dev Biol, 97*(2), 417-423.

Borlaug, B. A. (2014). The pathophysiology of heart failure with preserved ejection fraction. *Nat Rev Cardiol, 11*(9), 507-515. doi: 10.1038/nrcardio.2014.83

Borlaug, B. A., & Paulus, W. J. (2011). Heart failure with preserved ejection fraction: pathophysiology, diagnosis, and treatment. *Eur Heart J, 32*(6), 670-679. doi: 10.1093/eurheartj/ehq426

Boudina, S., & Abel, E. D. (2007). Diabetic cardiomyopathy revisited. *Circulation, 115*(25), 3213-3223. doi: 10.1161/circulationaha.106.679597

Bousquet, P., Feldman, J., & Schwartz, J. (1984). Central cardiovascular effects of alpha adrenergic drugs: differences between catecholamines and imidazolines. *J Pharmacol Exp Ther, 230*(1), 232-236.

Bricca, G., Dontenwill, M., Molines, A., Feldman, J., Belcourt, A., & Bousquet, P. (1989). The imidazoline preferring receptor: binding studies in bovine, rat and human brainstem. *Eur J Pharmacol, 162*(1), 1-9.

Bristow, M. R., Ginsburg, R., Umans, V., Fowler, M., Minobe, W., Rasmussen, R., . . . et al. (1986). Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle

contraction and selective beta 1-receptor down-regulation in heart failure. *Circ Res, 59*(3), 297-309.

Brodde, O. E., & Michel, M. C. (1999). Adrenergic and muscarinic receptors in the human heart. *Pharmacol Rev, 51*(4), 651-690.

Bronnum, H., Eskildsen, T., Andersen, D. C., Schneider, M., & Sheikh, S. P. (2013). IL-1beta suppresses TGF-beta-mediated myofibroblast differentiation in cardiac fibroblasts. *Growth Factors, 31*(3), 81-89. doi: 10.3109/08977194.2013.787994

Brunton, L. L., Chabner, B. A., & Knollmann, B. C. (2011). *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. (12$^{e}$ éd.).

Burgess, M. L., Terracio, L., Hirozane, T., & Borg, T. K. (2002). Differential integrin expression by cardiac fibroblasts from hypertensive and exercise-trained rat hearts. *Cardiovasc Pathol, 11*(2), 78-87.

Bustelo, X. R., Sauzeau, V., & Berenjeno, I. M. (2007). GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays, 29*(4), 356-370. doi: 10.1002/bies.20558

Cabassi, A., Dancelli, S., Pattoneri, P., Tirabassi, G., Quartieri, F., Moschini, L., . . . Borghetti, A. (2007). Characterization of myocardial hypertrophy in prehypertensive spontaneously hypertensive rats: interaction between adrenergic and nitrosative pathways. *J Hypertens, 25*(8), 1719-1730. doi: 10.1097/HJH.0b013e3281de72f0

Cacciapuoti, F. (2011). Molecular mechanisms of left ventricular hypertrophy (LVH) in systemic hypertension (SH)-possible therapeutic perspectives. *J Am Soc Hypertens, 5*(6), 449-455. doi: 10.1016/j.jash.2011.08.006

Campbell, N. R., McAlister, F. A., & Quan, H. (2013). Monitoring and evaluating efforts to control hypertension in Canada: why, how, and what it tells us needs to be done about current care gaps. *Can J Cardiol, 29*(5), 564-570. doi: 10.1016/j.cjca.2012.05.006

Carey, R. M. (2013). Newly discovered components and actions of the renin-angiotensin system. *Hypertension, 62*(5), 818-822. doi: 10.1161/hypertensionaha.113.01111

Carey, R. M., & Siragy, H. M. (2003). Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. *Endocr Rev, 24*(3), 261-271. doi: 10.1210/er.2003-0001

Carreno, J. E., Apablaza, F., Ocaranza, M. P., & Jalil, J. E. (2006). [Cardiac hypertrophy: molecular and cellular events]. *Rev Esp Cardiol, 59*(5), 473-486.

Carver, W., Nagpal, M. L., Nachtigal, M., Borg, T. K., & Terracio, L. (1991). Collagen expression in mechanically stimulated cardiac fibroblasts. *Circ Res, 69*(1), 116-122.

Chaggar, P. S., Malkin, C. J., Shaw, S. M., Williams, S. G., & Channer, K. S. (2009). Neuroendocrine effects on the heart and targets for therapeutic manipulation in heart failure. *Cardiovasc Ther, 27*(3), 187-193. doi: 10.1111/j.1755-5922.2009.00094.x

Champion, H. C., Georgakopoulos, D., Haldar, S., Wang, L., Wang, Y., & Kass, D. A. (2003). Robust adenoviral and adeno-associated viral gene transfer to the in vivo murine heart: application to study of phospholamban physiology. *Circulation, 108*(22), 2790-2797. doi: 10.1161/01.cir.0000096487.88897.9b

Chan, C. K., Burke, S. L., Zhu, H., Piletz, J. E., & Head, G. A. (2005). Imidazoline receptors associated with noradrenergic terminals in the rostral ventrolateral medulla mediate the hypotensive responses of moxonidine but not clonidine. *Neuroscience, 132*(4), 991-1007. doi: 10.1016/j.neuroscience.2005.01.032

Chang, L., Chiang, S. H., & Saltiel, A. R. (2004). Insulin signaling and the regulation of glucose transport. *Mol Med, 10*(7-12), 65-71. doi: 10.2119/2005-00029.Saltiel

Chatterjee, P. K., Hawksworth, G. M., & McLay, J. S. (1999). Cytokine-stimulated nitric oxide production in the human renal proximal tubule and its modulation by natriuretic peptides: A novel immunomodulatory mechanism? *Exp Nephrol, 7*(5-6), 438-448. doi: 20623

Chazova, I., & Schlaich, M. P. (2013). Improved Hypertension Control with the Imidazoline Agonist Moxonidine in a Multinational Metabolic Syndrome Population: Principal Results of the MERSY Study. *Int J Hypertens, 2013*, 541689. doi: 10.1155/2013/541689

Chen, Z., Jiang, H., Wan, Y., Bi, C., & Yuan, Y. (2012). H(2)O (2)-induced secretion of tumor necrosis factor-alpha evokes apoptosis of cardiac myocytes through reactive oxygen species-dependent activation of p38 MAPK. *Cytotechnology, 64*(1), 65-73. doi: 10.1007/s10616-011-9392-3

Chobanian, A. V., Bakris, G. L., Black, H. R., Cushman, W. C., Green, L. A., Izzo, J. L., Jr., . . . Roccella, E. J. (2003). Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension, 42*(6), 1206-1252. doi: 10.1161/01.HYP.0000107251.49515.c2

Chung, E. S., Packer, M., Lo, K. H., Fasanmade, A. A., & Willerson, J. T. (2003). Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: results of the anti-TNF Therapy Against Congestive Heart Failure (ATTACH) trial. *Circulation, 107*(25), 3133-3140. doi: 10.1161/01.cir.0000077913.60364.d2

Clark, K., Pankov, R., Travis, M. A., Askari, J. A., Mould, A. P., Craig, S. E., . . . Humphries, M. J. (2005). A specific alpha5beta1-integrin conformation promotes directional integrin translocation and fibronectin matrix formation. *J Cell Sci, 118*(Pt 2), 291-300. doi: 10.1242/jcs.01623

Coats, A. J. (1999). Heart Failure 99 -- the MOXCON story. *Int J Cardiol, 71*(2), 109-111.

Cohn, J. N., Pfeffer, M. A., Rouleau, J., Sharpe, N., Swedberg, K., Straub, M., . . . Wright, T. J. (2003). Adverse mortality effect of central sympathetic inhibition with sustained-release moxonidine in patients with heart failure (MOXCON). *Eur J Heart Fail, 5*(5), 659-667.

Communal, C., Singh, K., Pimentel, D. R., & Colucci, W. S. (1998). Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. *Circulation, 98*(13), 1329-1334.

Cuspidi, C., Negri, F., Muiesan, M. L., Capra, A., Lonati, L., Milan, A., . . . Morganti, A. (2011). Prevalence and severity of echocardiographic left ventricular hypertrophy in hypertensive patients in clinical practice. *Blood Press, 20*(1), 3-9. doi: 10.3109/08037051.2010.514713

D'Souza, A., Howarth, F. C., Yanni, J., Dobrzynski, H., Boyett, M. R., Adeghate, E., . . . Singh, J. (2014). Chronic effects of mild hyperglycaemia on left ventricle transcriptional profile and structural remodelling in the spontaneously type 2 diabetic Goto-Kakizaki rat. *Heart Fail Rev, 19*(1), 65-74. doi: 10.1007/s10741-013-9376-9

Danaei, G., Finucane, M. M., Lin, J. K., Singh, G. M., Paciorek, C. J., Cowan, M. J., . . . Ezzati, M. (2011). National, regional, and global trends in systolic blood pressure since

1980: systematic analysis of health examination surveys and epidemiological studies with 786 country-years and 5.4 million participants. *Lancet, 377*(9765), 568-577. doi: 10.1016/s0140-6736(10)62036-3

Das, A., Xi, L., & Kukreja, R. C. (2005). Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. *J Biol Chem, 280*(13), 12944-12955. doi: 10.1074/jbc.M404706200

Daugherty, S. L., Powers, J. D., Magid, D. J., Tavel, H. M., Masoudi, F. A., Margolis, K. L., . . . Ho, P. M. (2012). Incidence and prognosis of resistant hypertension in hypertensive patients. *Circulation, 125*(13), 1635-1642. doi: 10.1161/circulationaha.111.068064

Davies, P. F., Robotewskyj, A., & Griem, M. L. (1994). Quantitative studies of endothelial cell adhesion. Directional remodeling of focal adhesion sites in response to flow forces. *J Clin Invest, 93*(5), 2031-2038. doi: 10.1172/jci117197

de Bold, A. J., Borenstein, H. B., Veress, A. T., & Sonnenberg, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci, 28*(1), 89-94.

De La Pena, A., & De Castro, F. (1960). Structure and arrangement of the "macula densa" in the human kidney. *Urol Int, 10*, 171-178.

De Marchi, S. F., Allemann, Y., & Seiler, C. (2000). Relaxation in hypertrophic cardiomyopathy and hypertensive heart disease: relations between hypertrophy and diastolic function. *Heart, 83*(6), 678-684.

De Tommasi, E., Iacoviello, M., Romito, R., Ceconi, C., Guida, P., Massari, F., . . . Pitzalis, M. V. (2003). Comparison of the effect of valsartan and lisinopril on autonomic nervous system activity in chronic heart failure. *Am Heart J, 146*(5), E17. doi: 10.1016/s0002-8703(03)00366-1

Defer, N., Azroyan, A., Pecker, F., & Pavoine, C. (2007). TNFR1 and TNFR2 signaling interplay in cardiac myocytes. *J Biol Chem, 282*(49), 35564-35573. doi: 10.1074/jbc.M704003200

Dekleva, M., Dungen, H. D., Gelbrich, G., Incrot, S., Suzic Lazic, J., Pavlovic Kleut, M., . . . Waagstein, F. (2012). Beta blockers therapy is associated with improved left ventricular systolic function and sustained exercise capacity in elderly patients with heart failure. CIBIS-ELD sub-study. *Aging Clin Exp Res, 24*(6), 675-681. doi: 10.3275/8720

Del Ry, S., Cabiati, M., Vozzi, F., Battolla, B., Caselli, C., Forini, F., . . . Mattii, L. (2011). Expression of C-type natriuretic peptide and its receptor NPR-B in cardiomyocytes. *Peptides, 32*(8), 1713-1718. doi: 10.1016/j.peptides.2011.06.014

Derosa, G., Cicero, A. F., D'Angelo, A., Fogari, E., Salvadeo, S., Gravina, A., . . . Fogari, R. (2007). Metabolic and antihypertensive effects of moxonidine and moxonidine plus irbesartan in patients with type 2 diabetes mellitus and mild hypertension: a sequential, randomized, double-blind clinical trial. *Clin Ther, 29*(4), 602-610. doi: 10.1016/j.clinthera.2007.03.015

Devereux, R. B., Wachtell, K., Gerdts, E., Boman, K., Nieminen, M. S., Papademetriou, V., . . . Dahlof, B. (2004). Prognostic significance of left ventricular mass change during treatment of hypertension. *Jama, 292*(19), 2350-2356. doi: 10.1001/jama.292.19.2350

Dhingra, S., Sharma, A. K., Singla, D. K., & Singal, P. K. (2007). p38 and ERK1/2 MAPKs mediate the interplay of TNF-alpha and IL-10 in regulating oxidative stress and cardiac

myocyte apoptosis. *Am J Physiol Heart Circ Physiol, 293*(6), H3524-3531. doi: 10.1152/ajpheart.00919.2007

DiBona, G. F. (2002). Sympathetic nervous system and the kidney in hypertension. *Curr Opin Nephrol Hypertens, 11*(2), 197-200.

Dickstein, K., Manhenke, C., Aarsland, T., Kopp, U., McNay, J., & Wiltse, C. (1999). Acute hemodynamic and neurohumoral effects of moxonidine in congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. *Am J Cardiol, 83*(12), 1638-1644.

Dietz, J. R. (2005). Mechanisms of atrial natriuretic peptide secretion from the atrium. *Cardiovasc Res, 68*(1), 8-17. doi: 10.1016/j.cardiores.2005.06.008

Diez, J. (2008). Diagnosis and treatment of myocardial fibrosis in hypertensive heart disease. *Circ J, 72 Suppl A*, A8-12.

Diez, J., Fortuno, M. A., & Ravassa, S. (1998). Apoptosis in hypertensive heart disease. *Curr Opin Cardiol, 13*(5), 317-325.

Diez, J., Querejeta, R., Lopez, B., Gonzalez, A., Larman, M., & Martinez Ubago, J. L. (2002). Losartan-dependent regression of myocardial fibrosis is associated with reduction of left ventricular chamber stiffness in hypertensive patients. *Circulation, 105*(21), 2512-2517.

Ding, Y., Milosavljevic, T., & Alahari, S. K. (2008). Nischarin inhibits LIM kinase to regulate cofilin phosphorylation and cell invasion. *Mol Cell Biol, 28*(11), 3742-3756. doi: 10.1128/mcb.01832-07

DiNicolantonio, J. J., Lavie, C. J., Fares, H., Menezes, A. R., & O'Keefe, J. H. (2013). Meta-analysis of carvedilol versus beta 1 selective beta-blockers (atenolol, bisoprolol, metoprolol, and nebivolol). *Am J Cardiol, 111*(5), 765-769. doi: 10.1016/j.amjcard.2012.11.031

Doetschman, T., & Azhar, M. (2012). Cardiac-specific inducible and conditional gene targeting in mice. *Circ Res, 110*(11), 1498-1512. doi: 10.1161/circresaha.112.265066

Donahue, J. K., Kikkawa, K., Johns, D. C., Marban, E., & Lawrence, J. H. (1997). Ultrarapid, highly efficient viral gene transfer to the heart. *Proc Natl Acad Sci U S A, 94*(9), 4664-4668.

Dontenwill, M., Pascal, G., Piletz, J. E., Chen, M., Baldwin, J., Ronde, P., . . . Bousquet, P. (2003). IRAS, the human homologue of Nischarin, prolongs survival of transfected PC12 cells. *Cell Death Differ, 10*(8), 933-935. doi: 10.1038/sj.cdd.4401275

Dontenwill, M., Piletz, J. E., Chen, M., Baldwin, J., Pascal, G., Ronde, P., . . . Bousquetd, P. (2003). IRAS is an anti-apoptotic protein. *Ann N Y Acad Sci, 1009*, 400-412.

Dorn, G. W., 2nd. (2002). Adrenergic pathways and left ventricular remodeling. *J Card Fail, 8*(6 Suppl), S370-373. doi: 10.1054/jcaf.2002.129267

Dornas, W. C., & Silva, M. E. (2011). Animal models for the study of arterial hypertension. *J Biosci, 36*(4), 731-737.

Drawnel, F. M., Archer, C. R., & Roderick, H. L. (2013). The role of the paracrine/autocrine mediator endothelin-1 in regulation of cardiac contractility and growth. *Br J Pharmacol, 168*(2), 296-317. doi: 10.1111/j.1476-5381.2012.02195.x

Drexler, H. (1999). Nitric oxide synthases in the failing human heart: a doubled-edged sword? *Circulation, 99*(23), 2972-2975.

Drimal, J., Mislovicova, M., Ismail, A., & Moncek, F. (1999). Detrimental subtype-specific endothelin-1 (ET-1) signaling in myocardial cells: the ET(A) mediated proliferation and ET(B)receptor down-regulation. *Physiol Res, 48*(1), 9-19.

Duerrschmid, C., Crawford, J. R., Reineke, E., Taffet, G. E., Trial, J., Entman, M. L., & Haudek, S. B. (2013). TNF receptor 1 signaling is critically involved in mediating angiotensin-II-induced cardiac fibrosis. *J Mol Cell Cardiol, 57*, 59-67. doi: 10.1016/j.yjmcc.2013.01.006

Dupont, S., Maizel, J., Mentaverri, R., Chillon, J. M., Six, I., Giummelly, P., . . . Slama, M. (2012). The onset of left ventricular diastolic dysfunction in SHR rats is not related to hypertrophy or hypertension. *Am J Physiol Heart Circ Physiol, 302*(7), H1524-1532. doi: 10.1152/ajpheart.00955.2010

Dupuy, L., Urosevic, D., Greney, H., Quaglia, W., Pigini, M., Brasili, L., . . . Bousquet, P. (2004). I1 imidazoline receptor-mediated effects on apoptotic processes in PC12 cells. *Cell Death Differ, 11*(9), 1049-1052. doi: 10.1038/sj.cdd.4401447

Edwards, L., Fishman, D., Horowitz, P., Bourbon, N., Kester, M., & Ernsberger, P. (2001). The I1-imidazoline receptor in PC12 pheochromocytoma cells activates protein kinases C, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK). *J Neurochem, 79*(5), 931-940.

Edwards, L. P., Brown-Bryan, T. A., McLean, L., & Ernsberger, P. (2012). Pharmacological properties of the central antihypertensive agent, moxonidine. *Cardiovasc Ther, 30*(4), 199-208. doi: 10.1111/j.1755-5922.2011.00268.x

Eisner, D., Bode, E., Venetucci, L., & Trafford, A. (2013). Calcium flux balance in the heart. *J Mol Cell Cardiol, 58*, 110-117. doi: 10.1016/j.yjmcc.2012.11.017

Ekblom, B., & Hermansen, L. (1968). Cardiac output in athletes. *J Appl Physiol, 25*(5), 619-625.

El-Ayoubi, R., Gutkowska, J., Regunathan, S., & Mukaddam-Daher, S. (2002). Imidazoline receptors in the heart: characterization, distribution, and regulation. *J Cardiovasc Pharmacol, 39*(6), 875-883.

El-Ayoubi, R., Menaouar, A., Gutkowska, J., & Mukaddam-Daher, S. (2003). Normalization of up-regulated cardiac imidazoline I(1)-receptors and natriuretic peptides by chronic treatment with moxonidine in spontaneously hypertensive rats. *Ann N Y Acad Sci, 1009*, 274-278.

El-Ayoubi, R., Menaouar, A., Gutkowska, J., & Mukaddam-Daher, S. (2004). Imidazoline receptors but not alpha 2-adrenoceptors are regulated in spontaneously hypertensive rat heart by chronic moxonidine treatment. *J Pharmacol Exp Ther, 310*(2), 446-451. doi: 10.1124/jpet.104.067595

El-Ayoubi, R., Menaouar, A., Gutkowska, J., & Mukaddam-Daher, S. (2005). Urinary responses to acute moxonidine are inhibited by natriuretic peptide receptor antagonist. *Br J Pharmacol, 145*(1), 50-56. doi: 10.1038/sj.bjp.0706146

El-Mas, M. M., El-Gowelli, H. M., Ghazal, A. R., Harraz, O. F., & Mohy El-Din, M. M. (2009). Facilitation of central imidazoline I(1)-site/extracellular signal-regulated kinase/p38 mitogen-activated protein kinase signalling mediates the hypotensive effect of ethanol in rats with acute renal failure. *Br J Pharmacol, 158*(6), 1629-1640. doi: 10.1111/j.1476-5381.2009.00444.x

El-Omar, M. M., Yang, Z. K., Phillips, A. O., & Shah, A. M. (2004). Cardiac dysfunction in the Goto-Kakizaki rat. A model of type II diabetes mellitus. *Basic Res Cardiol, 99*(2), 133-141. doi: 10.1007/s00395-004-0440-4

El Andalousi, J., Li, Y., & Anand-Srivastava, M. B. (2013). Natriuretic peptide receptor-C agonist attenuates the expression of cell cycle proteins and proliferation of vascular smooth muscle cells from spontaneously hypertensive rats: role of Gi proteins and MAPkinase/PI3kinase signaling. *PLoS One, 8*(10), e76183. doi: 10.1371/journal.pone.0076183

Elks, C. M., Mariappan, N., Haque, M., Guggilam, A., Majid, D. S., & Francis, J. (2009). Chronic NF-{kappa}B blockade reduces cytosolic and mitochondrial oxidative stress and attenuates renal injury and hypertension in SHR. *Am J Physiol Renal Physiol, 296*(2), F298-305. doi: 10.1152/ajprenal.90628.2008

Elsasser, A., Suzuki, K., & Schaper, J. (2000). Unresolved issues regarding the role of apoptosis in the pathogenesis of ischemic injury and heart failure. *J Mol Cell Cardiol, 32*(5), 711-724. doi: 10.1006/jmcc.2000.1125

Employment and Social Development Canada. (2014, 22/04/2014). Canadians in Context-Aging population. Repéré le 22/04/2014 2014 à http://www4.hrsdc.gc.ca/.3ndic.1t.4r@-eng.jsp?iid=33

Ernsberger, P. (1998). Arachidonic acid release from PC12 pheochromocytoma cells is regulated by I1-imidazoline receptors. *J Auton Nerv Syst, 72*(2-3), 147-154.

Ernsberger, P. (1999). The I1-imidazoline receptor and its cellular signaling pathways. *Ann N Y Acad Sci, 881*, 35-53.

Ernsberger, P. (2000). Pharmacology of moxonidine: an I1-imidazoline receptor agonist. *J Cardiovasc Pharmacol, 35*(7 Suppl 4), S27-41.

Ernsberger, P., Damon, T. H., Graff, L. M., Schafer, S. G., & Christen, M. O. (1993). Moxonidine, a centrally acting antihypertensive agent, is a selective ligand for I1-imidazoline sites. *J Pharmacol Exp Ther, 264*(1), 172-182.

Ernsberger, P., Giuliano, R., Willette, R. N., Granata, A. R., & Reis, D. J. (1988). Hypotensive action of clonidine analogues correlates with binding affinity at imidazole and not alpha-2-adrenergic receptors in the rostral ventrolateral medulla. *J Hypertens Suppl, 6*(4), S554-557.

Ernsberger, P., Ishizuka, T., Liu, S., Farrell, C. J., Bedol, D., Koletsky, R. J., & Friedman, J. E. (1999). Mechanisms of antihyperglycemic effects of moxonidine in the obese spontaneously hypertensive Koletsky rat (SHROB). *J Pharmacol Exp Ther, 288*(1), 139-147.

Esposito, G., Rapacciuolo, A., Naga Prasad, S. V., Takaoka, H., Thomas, S. A., Koch, W. J., & Rockman, H. A. (2002). Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. *Circulation, 105*(1), 85-92.

Evans, E. A., & Calderwood, D. A. (2007). Forces and bond dynamics in cell adhesion. *Science, 316*(5828), 1148-1153. doi: 10.1126/science.1137592

Fenton, C., Keating, G. M., & Lyseng-Williamson, K. A. (2006). Moxonidine: a review of its use in essential hypertension. *Drugs, 66*(4), 477-496.

Ferrara, L. A., Vaccaro, O., Cardoni, O., Mancini, M., & Zanchetti, A. (2004). Arterial hypertension increases left ventricular mass: role of tight blood pressure control. *J Hum Hypertens, 18*(9), 637-642. doi: 10.1038/sj.jhh.1001698

Fine, D., Meiselas, L. E., & Auerbach, T. (1958). The effect of acute hypovolemia on the release of aldosterone and on the renal excretion of sodium. *J Clin Invest, 37*(2), 232-243. doi: 10.1172/jci103602

Fiscus, R. R., Tu, A. W., & Chew, S. B. (2001). Natriuretic peptides inhibit apoptosis and prolong the survival of serum-deprived PC12 cells. *Neuroreport, 12*(2), 185-189.

Fisher, J. P., & Fadel, P. J. (2010). Therapeutic strategies for targeting excessive central sympathetic activation in human hypertension. *Exp Physiol, 95*(5), 572-580. doi: 10.1113/expphysiol.2009.047332

Floras, J. S. (2002). The "unsympathetic" nervous system of heart failure. *Circulation, 105*(15), 1753-1755.

Foo, R. S., Mani, K., & Kitsis, R. N. (2005). Death begets failure in the heart. *J Clin Invest, 115*(3), 565-571. doi: 10.1172/jci24569

Fournier, D., Luft, F. C., Bader, M., Ganten, D., & Andrade-Navarro, M. A. (2012). Emergence and evolution of the renin-angiotensin-aldosterone system. *J Mol Med (Berl), 90*(5), 495-508. doi: 10.1007/s00109-012-0894-z

Fox, K., Ford, I., Steg, P. G., Tendera, M., & Ferrari, R. (2008). Ivabradine for patients with stable coronary artery disease and left-ventricular systolic dysfunction (BEAUTIFUL): a randomised, double-blind, placebo-controlled trial. *Lancet, 372*(9641), 807-816. doi: 10.1016/s0140-6736(08)61170-8

Franco, V., Chen, Y. F., Oparil, S., Feng, J. A., Wang, D., Hage, F., & Perry, G. (2004). Atrial natriuretic peptide dose-dependently inhibits pressure overload-induced cardiac remodeling. *Hypertension, 44*(5), 746-750. doi: 10.1161/01.HYP.0000144801.09557.4c

Friedman, S. M., Friedman, C. L., & Nakashima, M. (1958). Effects of aldosterone on blood pressure and electrolyte distribution in the rat. *Am J Physiol, 195*(3), 621-627.

Frohlich, E. D., Gonzalez, A., & Diez, J. (2011). Hypertensive left ventricular hypertrophy risk: beyond adaptive cardiomyocytic hypertrophy. *J Hypertens, 29*(1), 17-26. doi: 10.1097/HJH.0b013e328340d787

Fu, Y. C., Chi, C. S., Yin, S. C., Hwang, B., Chiu, Y. T., & Hsu, S. L. (2004). Norepinephrine induces apoptosis in neonatal rat cardiomyocytes through a reactive oxygen species-TNF alpha-caspase signaling pathway. *Cardiovasc Res, 62*(3), 558-567. doi: 10.1016/j.cardiores.2004.01.039

Fukuta, H., & Little, W. C. (2008). The cardiac cycle and the physiologic basis of left ventricular contraction, ejection, relaxation, and filling. *Heart Fail Clin, 4*(1), 1-11. doi: 10.1016/j.hfc.2007.10.004

Gailit, J., Xu, J., Bueller, H., & Clark, R. A. (1996). Platelet-derived growth factor and inflammatory cytokines have differential effects on the expression of integrins alpha 1 beta 1 and alpha 5 beta 1 by human dermal fibroblasts in vitro. *J Cell Physiol, 169*(2), 281-289. doi: 10.1002/(sici)1097-4652(199611)169:2<281::aid-jcp7>3.0.co;2-k

Gan, X. T., Chakrabarti, S., & Karmazyn, M. (2003). Increased endothelin-1 and endothelin receptor expression in myocytes of ischemic and reperfused rat hearts and ventricular myocytes exposed to ischemic conditions and its inhibition by nitric oxide generation. *Can J Physiol Pharmacol, 81*(2), 105-113. doi: 10.1139/y03-030

Gao, L., Wang, W., Li, Y. L., Schultz, H. D., Liu, D., Cornish, K. G., & Zucker, I. H. (2005). Sympathoexcitation by central ANG II: roles for AT1 receptor upregulation and

NAD(P)H oxidase in RVLM. *Am J Physiol Heart Circ Physiol, 288*(5), H2271-2279. doi: 10.1152/ajpheart.00949.2004

Garcia-Sevilla, J., Miralles, A., Sastre, M., Escriba, P. V., Olmos, G., & Meana, J. J. (1995). I2-imidazoline receptors in the healthy and pathologic human brain. *Ann N Y Acad Sci, 763*, 178-193.

George, M., Rajaram, M., Shanmugam, E., & Vijayakumar, T. M. (2014). Novel drug targets in clinical development for heart failure. *Eur J Clin Pharmacol*. doi: 10.1007/s00228-014-1671-4

Gertz, E. W. (1972). Cardiomyopathic Syrian hamster: a possible model of human disease. *Prog Exp Tumor Res, 16*, 242-260.

Gilbert, E. M., Abraham, W. T., Olsen, S., Hattler, B., White, M., Mealy, P., . . . Bristow, M. R. (1996). Comparative hemodynamic, left ventricular functional, and antiadrenergic effects of chronic treatment with metoprolol versus carvedilol in the failing heart. *Circulation, 94*(11), 2817-2825.

Girard, P. R., & Nerem, R. M. (1995). Shear stress modulates endothelial cell morphology and F-actin organization through the regulation of focal adhesion-associated proteins. *J Cell Physiol, 163*(1), 179-193. doi: 10.1002/jcp.1041630121

Goldenberg, I., Grossman, E., Jacobson, K. A., Shneyvays, V., & Shainberg, A. (2001). Angiotensin II-induced apoptosis in rat cardiomyocyte culture: a possible role of AT1 and AT2 receptors. *J Hypertens, 19*(9), 1681-1689.

Gower, W. R., Jr., Dietz, J. R., Vesely, D. L., Finley, C. L., Skolnick, K. A., Fabri, P. J., . . . Chalfant, C. E. (1994). Atrial natriuretic peptide gene expression in the rat gastrointestinal tract. *Biochem Biophys Res Commun, 202*(1), 562-570. doi: 10.1006/bbrc.1994.1965

Grassi, G. (2013). The 'renaissance era' of sympathomodulatory interventions in the treatment of hypertension-related congestive heart failure. *J Hypertens, 31*(11), 2133-2135. doi: 10.1097/HJH.0b013e32836541ae

Grassi, G., Seravalle, G., & Quarti-Trevano, F. (2010). The 'neuroadrenergic hypothesis' in hypertension: current evidence. *Exp Physiol, 95*(5), 581-586. doi: 10.1113/expphysiol.2009.047381

Gray, M. O., Long, C. S., Kalinyak, J. E., Li, H. T., & Karliner, J. S. (1998). Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta 1 and endothelin-1 from fibroblasts. *Cardiovasc Res, 40*(2), 352-363.

Gullestad, L., Ueland, T., Vinge, L. E., Finsen, A., Yndestad, A., & Aukrust, P. (2012). Inflammatory cytokines in heart failure: mediators and markers. *Cardiology, 122*(1), 23-35. doi: 10.1159/000338166

Gupta, S., & Sharma, B. (2014). Pharmacological benefit of I(1)-imidazoline receptors activation and nuclear factor kappa-B (NF-kappaB) modulation in experimental Huntington's disease. *Brain Res Bull, 102*, 57-68. doi: 10.1016/j.brainresbull.2014.02.007

Hackam, D. G., Quinn, R. R., Ravani, P., Rabi, D. M., Dasgupta, K., Daskalopoulou, S. S., . . . Padwal, R. S. (2013). The 2013 Canadian Hypertension Education Program recommendations for blood pressure measurement, diagnosis, assessment of risk, prevention, and treatment of hypertension. *Can J Cardiol, 29*(5), 528-542. doi: 10.1016/j.cjca.2013.01.005

Haczynski, J., Flasinski, J., Przewlocka-Kosmala, M., & Spring, A. (2001). Effect of moxonidine on left ventricular hypertrophy in hypertensive patients. *Journal of Clinical and Basic Cardiology, 4*(1), 61-65.

Haggart, C. R., Ames, E. G., Lee, J. K., & Holmes, J. W. (2014). Effects of stretch and shortening on gene expression in intact myocardium. *Physiol Genomics, 46*(2), 57-65. doi: 10.1152/physiolgenomics.00103.2013

Halaris, A., & Piletz, J. E. (2003). Relevance of imidazoline receptors and agmatine to psychiatry: a decade of progress. *Ann N Y Acad Sci, 1009*, 1-20.

Halley, C. M., Houghtaling, P. L., Khalil, M. K., Thomas, J. D., & Jaber, W. A. (2011). Mortality rate in patients with diastolic dysfunction and normal systolic function. *Arch Intern Med, 171*(12), 1082-1087. doi: 10.1001/archinternmed.2011.244

Hamilton, C. A. (1995). Imidazoline receptors, subclassification, and drug-induced regulation. *Ann N Y Acad Sci, 763*, 57-65.

Hannun, Y. A., & Obeid, L. M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol, 9*(2), 139-150. doi: 10.1038/nrm2329

Harding, P., Carretero, O. A., & LaPointe, M. C. (1995). Effects of interleukin-1 beta and nitric oxide on cardiac myocytes. *Hypertension, 25*(3), 421-430.

Hart, D. L., Heidkamp, M. C., Iyengar, R., Vijayan, K., Szotek, E. L., Barakat, J. A., . . . Samarel, A. M. (2008). CRNK gene transfer improves function and reverses the myosin heavy chain isoenzyme switch during post-myocardial infarction left ventricular remodeling. *J Mol Cell Cardiol, 45*(1), 93-105. doi: 10.1016/j.yjmcc.2008.04.002

Haudek, S. B., Taffet, G. E., Schneider, M. D., & Mann, D. L. (2007). TNF provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways. *J Clin Invest, 117*(9), 2692-2701. doi: 10.1172/jci29134

Hausberg, M., Tokmak, F., Pavenstadt, H., Kramer, B. K., & Rump, L. C. (2010). Effects of moxonidine on sympathetic nerve activity in patients with end-stage renal disease. *J Hypertens, 28*(9), 1920-1927. doi: 10.1097/HJH.0b013e32833c2100

Hausdorff, J. M., Clark, B. A., Shannon, R. P., Elahi, D., & Wei, J. Y. (1995). Hypotensive response to atrial natriuretic peptide administration is enhanced with age. *J Gerontol A Biol Sci Med Sci, 50*(3), M169-172.

Haxhiu, M. A., Dreshaj, I., Schafer, S. G., & Ernsberger, P. (1994). Selective antihypertensive action of moxonidine is mediated mainly by I1-imidazoline receptors in the rostral ventrolateral medulla. *J Cardiovasc Pharmacol, 24 Suppl 1*, S1-8.

He, Y. H., Zhang, H. N., Zhang, G. P., Hou, N., Xiao, Q., Huang, Y., . . . Luo, J. D. (2011). A physiological concentration of glucocorticoid inhibits the pro-inflammatory cytokine-induced proliferation of adult rat cardiac fibroblasts: roles of extracellular signal-regulated kinase 1/2 and nuclear factor-kappaB. *Clin Exp Pharmacol Physiol, 38*(11), 739-746. doi: 10.1111/j.1440-1681.2011.05581.x

Heart and Stroke Fundation of Canada. (2013). Statistics. Repéré le 29/07/2013 2013 à http://www.heartandstroke.com/site/c.ikIQLcMWJtE/b.3483991/k.34A8/Statistics.htm

Hedayat, M., Mahmoudi, M. J., Rose, N. R., & Rezaei, N. (2010). Proinflammatory cytokines in heart failure: double-edged swords. *Heart Fail Rev, 15*(6), 543-562. doi: 10.1007/s10741-010-9168-4

Hein, L., Altman, J. D., & Kobilka, B. K. (1999). Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission. *Nature, 402*(6758), 181-184. doi: 10.1038/46040

Hernandez, G., Lal, H., Fidalgo, M., Guerrero, A., Zalvide, J., Force, T., & Pombo, C. M. (2011). A novel cardioprotective p38-MAPK/mTOR pathway. *Exp Cell Res, 317*(20), 2938-2949. doi: 10.1016/j.yexcr.2011.09.011

Hidalgo, C., & Granzier, H. (2013). Tuning the molecular giant titin through phosphorylation: role in health and disease. *Trends Cardiovasc Med, 23*(5), 165-171. doi: 10.1016/j.tcm.2012.10.005

Higuchi, Y., Otsu, K., Nishida, K., Hirotani, S., Nakayama, H., Yamaguchi, O., . . . Hori, M. (2002). Involvement of reactive oxygen species-mediated NF-kappa B activation in TNF-alpha-induced cardiomyocyte hypertrophy. *J Mol Cell Cardiol, 34*(2), 233-240. doi: 10.1006/jmcc.2001.1505

Hilal-Dandan, R., Urasawa, K., & Brunton, L. L. (1992). Endothelin inhibits adenylate cyclase and stimulates phosphoinositide hydrolysis in adult cardiac myocytes. *J Biol Chem, 267*(15), 10620-10624.

Hiraoka, E., Kawashima, S., Takahashi, T., Rikitake, Y., Kitamura, T., Ogawa, W., & Yokoyama, M. (2001). TNF-alpha induces protein synthesis through PI3-kinase-Akt/PKB pathway in cardiac myocytes. *Am J Physiol Heart Circ Physiol, 280*(4), H1861-1868.

Hofstaetter, B., Taimor, G., Inserte, J., Garcia-Dorado, D., & Piper, H. M. (2002). Inhibition of apoptotic responses after ischemic stress in isolated hearts and cardiomyocytes. *Basic Res Cardiol, 97*(6), 479-488. doi: 10.1007/s003950200053

Holmer, S. R., Kaissling, B., Putnik, K., Pfeifer, M., Kramer, B. K., Riegger, G. A., & Kurtz, A. (1997). Beta-adrenergic stimulation of renin expression in vivo. *J Hypertens, 15*(12 Pt 1), 1471-1479.

Honsho, S., Nishikawa, S., Amano, K., Zen, K., Adachi, Y., Kishita, E., . . . Matsubara, H. (2009). Pressure-mediated hypertrophy and mechanical stretch induces IL-1 release and subsequent IGF-1 generation to maintain compensative hypertrophy by affecting Akt and JNK pathways. *Circ Res, 105*(11), 1149-1158. doi: 10.1161/circresaha.109.208199

Hordijk, P. L. (2006). Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res, 98*(4), 453-462. doi: 10.1161/01.RES.0000204727.46710.5e

Horio, T., Kohno, M., & Takeda, T. (1993). Cosecretion of atrial and brain natriuretic peptides stimulated by endothelin-1 from cultured rat atrial and ventricular cardiocytes. *Metabolism, 42*(1), 94-96.

Horio, T., Nishikimi, T., Yoshihara, F., Nagaya, N., Matsuo, H., Takishita, S., & Kangawa, K. (1998). Production and secretion of adrenomedullin in cultured rat cardiac myocytes and nonmyocytes: stimulation by interleukin-1beta and tumor necrosis factor-alpha. *Endocrinology, 139*(11), 4576-4580. doi: 10.1210/endo.139.11.6306

Hosoda, C., Tanoue, A., Shibano, M., Tanaka, Y., Hiroyama, M., Koshimizu, T. A., . . . Koike, K. (2005). Correlation between vasoconstrictor roles and mRNA expression of alpha1-adrenoceptor subtypes in blood vessels of genetically engineered mice. *Br J Pharmacol, 146*(3), 456-466. doi: 10.1038/sj.bjp.0706325

Houser, S. R., Margulies, K. B., Murphy, A. M., Spinale, F. G., Francis, G. S., Prabhu, S. D., . . . Koch, W. J. (2012). Animal models of heart failure: a scientific statement from the

American Heart Association. *Circ Res, 111*(1), 131-150. doi: 10.1161/RES.0b013e3182582523

Hu, Y., Li, T., Wang, Y., Li, J., Guo, L., Wu, M., . . . Li, Y. (2009). Tollip attenuated the hypertrophic response of cardiomyocytes induced by IL-1beta. *Front Biosci (Landmark Ed), 14*, 2747-2756.

Hunt, S. A., Abraham, W. T., Chin, M. H., Feldman, A. M., Francis, G. S., Ganiats, T. G., . . . Yancy, C. W. (2009). 2009 Focused update incorporated into the ACC/AHA 2005 Guidelines for the Diagnosis and Management of Heart Failure in Adults A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines Developed in Collaboration With the International Society for Heart and Lung Transplantation. *J Am Coll Cardiol, 53*(15), e1-e90. doi: 10.1016/j.jacc.2008.11.013

Huttenlocher, A., Sandborg, R. R., & Horwitz, A. F. (1995). Adhesion in cell migration. *Curr Opin Cell Biol, 7*(5), 697-706.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell, 69*(1), 11-25.

Iaizzo, P. A. (2009). *Handbook of cardiac anatomy, physiology, and devices*. (2e éd.).

Ichiki, Y., Kitamura, K., Kangawa, K., Kawamoto, M., Matsuo, H., & Eto, T. (1994). Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. *FEBS Lett, 338*(1), 6-10.

Iimura, O., Kusano, E., Homma, S., Takeda, S., Ikeda, U., Shimada, K., & Asano, Y. (1998). Atrial natriuretic peptide enhances IL-1 beta-stimulated nitric oxide production in cultured rat vascular smooth muscle cells. *Kidney Blood Press Res, 21*(1), 36-41.

Ikeda, S., Hamada, M., & Hiwada, K. (1999). Contribution of non-cardiomyocyte apoptosis to cardiac remodelling that occurs in the transition from compensated hypertrophy to heart failure in spontaneously hypertensive rats. *Clin Sci (Lond), 97*(2), 239-246.

Ing, D. J., Zang, J., Dzau, V. J., Webster, K. A., & Bishopric, N. H. (1999). Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. *Circ Res, 84*(1), 21-33.

Irzmanski, R., Barylski, M., Banach, M., Piechota, M., Kowalski, J., Cierniewski, C., & Pawlicki, L. (2007). The concentration of atrial and brain natriuretic peptide in patients with idiopathic hypertension. *Med Sci Monit, 13*(10), CR449-456.

Ivanov, T. R., Jones, J. C., Dontenwill, M., Bousquet, P., & Piletz, J. E. (1998). Characterization of a partial cDNA clone detected by imidazoline receptor-selective antisera. *J Auton Nerv Syst, 72*(2-3), 98-110.

Ivanov, T. R., Zhu, H., Regunathan, S., Reis, D. J., Dontenwill, M., Vonthron, C., . . . Piletz, J. E. (1998). Co-detection by two imidazoline receptor protein antisera of a novel 85 kilodalton protein. *Biochem Pharmacol, 55*(5), 649-655.

Ivey, M. E., Osman, N., & Little, P. J. (2008). Endothelin-1 signalling in vascular smooth muscle: pathways controlling cellular functions associated with atherosclerosis. *Atherosclerosis, 199*(2), 237-247. doi: 10.1016/j.atherosclerosis.2008.03.006

Iwai-Kanai, E., Hasegawa, K., Araki, M., Kakita, T., Morimoto, T., & Sasayama, S. (1999). alpha- and beta-adrenergic pathways differentially regulate cell type-specific apoptosis in rat cardiac myocytes. *Circulation, 100*(3), 305-311.

Iwai-Kanai, E., Hasegawa, K., Sawamura, T., Fujita, M., Yanazume, T., Toyokuni, S., . . . Sasayama, S. (2001). Activation of lectin-like oxidized low-density lipoprotein

receptor-1 induces apoptosis in cultured neonatal rat cardiac myocytes. *Circulation, 104*(24), 2948-2954.

Jain, P., Baranwal, S., Dong, S., Struckhoff, A. P., Worthylake, R. A., & Alahari, S. K. (2013). Integrin-binding protein nischarin interacts with tumor suppressor liver kinase B1 (LKB1) to regulate cell migration of breast epithelial cells. *J Biol Chem, 288*(22), 15495-15509. doi: 10.1074/jbc.M112.418103

James, P. A., Oparil, S., Carter, B. L., Cushman, W. C., Dennison-Himmelfarb, C., Handler, J., . . . Ortiz, E. (2014). 2014 evidence-based guideline for the management of high blood pressure in adults: report from the panel members appointed to the Eighth Joint National Committee (JNC 8). *Jama, 311*(5), 507-520. doi: 10.1001/jama.2013.284427

Jane-Lise, S., Corda, S., Chassagne, C., & Rappaport, L. (2000). The extracellular matrix and the cytoskeleton in heart hypertrophy and failure. *Heart Fail Rev, 5*(3), 239-250. doi: 10.1023/a:1009857403356

Jugdutt, B. I., & Amy, R. W. (1986). Healing after myocardial infarction in the dog: changes in infarct hydroxyproline and topography. *J Am Coll Cardiol, 7*(1), 91-102.

Kaganovsky, E., Belkin, V., Barhum, Y., Schaper, J., Schaper, W., & Kessler-Icekson, G. (2001). Occurrence and distribution of atrial natriuretic peptide-containing cells in the left ventricle of hypertensive rats. Effect of antihypertensive treatment. *Cell Tissue Res, 303*(1), 57-67.

Kalant, H., Grant, D. M., & Mitchell, J. (2007). *Principles of medical pharmacology*. (7$^{e}$ éd.). Saunders.

Kannel, W. B., Castelli, W. P., McNamara, P. M., McKee, P. A., & Feinleib, M. (1972). Role of blood pressure in the development of congestive heart failure. The Framingham study. *N Engl J Med, 287*(16), 781-787. doi: 10.1056/nejm197210192871601

Kannel, W. B., Kannel, C., Paffenbarger, R. S., Jr., & Cupples, L. A. (1987). Heart rate and cardiovascular mortality: the Framingham Study. *Am Heart J, 113*(6), 1489-1494.

Karliner, J. S., Honbo, N., Summers, K., Gray, M. O., & Goetzl, E. J. (2001). The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. *J Mol Cell Cardiol, 33*(9), 1713-1717. doi: 10.1006/jmcc.2001.1429

Kasama, S., Toyama, T., Hatori, T., Sumino, H., Kumakura, H., Takayama, Y., . . . Kurabayashi, M. (2007). Effects of intravenous atrial natriuretic peptide on cardiac sympathetic nerve activity and left ventricular remodeling in patients with first anterior acute myocardial infarction. *J Am Coll Cardiol, 49*(6), 667-674. doi: 10.1016/j.jacc.2006.09.048

Kato, T., Muraski, J., Chen, Y., Tsujita, Y., Wall, J., Glembotski, C. C., . . . Sussman, M. A. (2005). Atrial natriuretic peptide promotes cardiomyocyte survival by cGMP-dependent nuclear accumulation of zyxin and Akt. *J Clin Invest, 115*(10), 2716-2730. doi: 10.1172/jci24280

Kaya, D., Barutcu, I., Esen, A. M., Celik, A., & Onrat, E. (2010). Acute effects of moxonidine on cardiac autonomic modulation. *Pacing Clin Electrophysiol, 33*(8), 929-933. doi: 10.1111/j.1540-8159.2010.02727.x

Kemme, M. J., vd Post, J. P., Schoemaker, R. C., Straub, M., Cohen, A. F., & van Gerven, J. M. (2003). Central nervous system effects of moxonidine experimental sustained release formulation in patients with mild to moderate essential hypertension. *Br J Clin Pharmacol, 55*(6), 518-525.

Kemp, C. D., & Conte, J. V. (2012). The pathophysiology of heart failure. *Cardiovasc Pathol, 21*(5), 365-371. doi: 10.1016/j.carpath.2011.11.007

Khan, N. A., Herman, R. J., Quinn, R. R., Rabkin, S. W., Ravani, P., Tobe, S. W., . . . Padwal, R. S. (2014). Renal denervation therapy for the treatment of resistant hypertension: a position statement by the Canadian Hypertension Education Program. *Can J Cardiol, 30*(1), 16-21. doi: 10.1016/j.cjca.2013.07.008

Khayat, A., Gonda, S., Sen, S., & Smeby, R. R. (1981). Responses of juxtaglomerular cell suspensions to various stimuli. *Hypertension, 3*(2), 157-167.

Kiemer, A. K., Vollmar, A. M., Bilzer, M., Gerwig, T., & Gerbes, A. L. (2000). Atrial natriuretic peptide reduces expression of TNF-alpha mRNA during reperfusion of the rat liver upon decreased activation of NF-kappaB and AP-1. *J Hepatol, 33*(2), 236-246.

Kleinbongard, P., Heusch, G., & Schulz, R. (2010). TNFalpha in atherosclerosis, myocardial ischemia/reperfusion and heart failure. *Pharmacol Ther, 127*(3), 295-314. doi: 10.1016/j.pharmthera.2010.05.002

Kobe, B., & Kajava, A. V. (2001). The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol, 11*(6), 725-732.

Koda, M., Sakamoto, A., & Ogawa, R. (2005). Effects of atrial natriuretic peptide at a low dose on water and electrolyte metabolism during general anesthesia. *J Clin Anesth, 17*(1), 3-7. doi: 10.1016/j.jclinane.2004.03.006

Konstam, V., Moser, D. K., & De Jong, M. J. (2005). Depression and anxiety in heart failure. *J Card Fail, 11*(6), 455-463. doi: 10.1016/j.cardfail.2005.03.006

Konstandin, M. H., Toko, H., Gastelum, G. M., Quijada, P., De La Torre, A., Quintana, M., . . . Sussman, M. A. (2013). Fibronectin is essential for reparative cardiac progenitor cell response after myocardial infarction. *Circ Res, 113*(2), 115-125. doi: 10.1161/circresaha.113.301152

Konstandin, M. H., Volkers, M., Collins, B., Quijada, P., Quintana, M., De La Torre, A., . . . Sussman, M. A. (2013). Fibronectin contributes to pathological cardiac hypertrophy but not physiological growth. *Basic Res Cardiol, 108*(5), 375. doi: 10.1007/s00395-013-0375-8

Konstantinidis, K., Whelan, R. S., & Kitsis, R. N. (2012). Mechanisms of cell death in heart disease. *Arterioscler Thromb Vasc Biol, 32*(7), 1552-1562. doi: 10.1161/atvbaha.111.224915

Krishnamurthy, P., Subramanian, V., Singh, M., & Singh, K. (2007). Beta1 integrins modulate beta-adrenergic receptor-stimulated cardiac myocyte apoptosis and myocardial remodeling. *Hypertension, 49*(4), 865-872. doi: 10.1161/01.hyp.0000258703.36986.13

Kristal-Boneh, E., Silber, H., Harari, G., & Froom, P. (2000). The association of resting heart rate with cardiovascular, cancer and all-cause mortality. Eight year follow-up of 3527 male Israeli employees (the CORDIS Study). *Eur Heart J, 21*(2), 116-124. doi: 10.1053/euhj.1999.1741

Krown, K. A., Page, M. T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K. L., . . . Sabbadini, R. A. (1996). Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *J Clin Invest, 98*(12), 2854-2865. doi: 10.1172/jci119114

Kubota, T., Miyagishima, M., Frye, C. S., Alber, S. M., Bounoutas, G. S., Kadokami, T., . . . Feldman, A. M. (2001). Overexpression of tumor necrosis factor- alpha activates both

anti- and pro-apoptotic pathways in the myocardium. *J Mol Cell Cardiol, 33*(7), 1331-1344. doi: 10.1006/jmcc.2001.1393

Kuppers, H. E., Jager, B. A., Luszick, J. H., Grave, M. A., Hughes, P. R., & Kaan, E. C. (1997). Placebo-controlled comparison of the efficacy and tolerability of once-daily moxonidine and enalapril in mild-to-moderate essential hypertension. *J Hypertens, 15*(1), 93-97.

Kurisu, S., Ozono, R., Oshima, T., Kambe, M., Ishida, T., Sugino, H., . . . Matsubara, H. (2003). Cardiac angiotensin II type 2 receptor activates the kinin/NO system and inhibits fibrosis. *Hypertension, 41*(1), 99-107.

Kurtz, A. (2012). Control of renin synthesis and secretion. *Am J Hypertens, 25*(8), 839-847. doi: 10.1038/ajh.2011.246

Kuwahara, F., Kai, H., Tokuda, K., Niiyama, H., Tahara, N., Kusaba, K., . . . Imaizumi, T. (2003). Roles of intercellular adhesion molecule-1 in hypertensive cardiac remodeling. *Hypertension, 41*(3 Pt 2), 819-823. doi: 10.1161/01.hyp.0000056108.73219.0a

Kyoi, S., Otani, H., Matsuhisa, S., Akita, Y., Tatsumi, K., Enoki, C., . . . Iwasaka, T. (2006). Opposing effect of p38 MAP kinase and JNK inhibitors on the development of heart failure in the cardiomyopathic hamster. *Cardiovasc Res, 69*(4), 888-898. doi: 10.1016/j.cardiores.2005.11.015

Lai, K. B., Sanderson, J. E., & Yu, C. M. (2009). Suppression of collagen production in norepinephrine stimulated cardiac fibroblasts culture: differential effect of alpha and beta-adrenoreceptor antagonism. *Cardiovasc Drugs Ther, 23*(4), 271-280. doi: 10.1007/s10557-009-6183-6

Lam, C. S., Lyass, A., Kraigher-Krainer, E., Massaro, J. M., Lee, D. S., Ho, J. E., . . . Vasan, R. S. (2011). Cardiac dysfunction and noncardiac dysfunction as precursors of heart failure with reduced and preserved ejection fraction in the community. *Circulation, 124*(1), 24-30. doi: 10.1161/circulationaha.110.979203

Lam, C. S., Shah, A. M., Borlaug, B. A., Cheng, S., Verma, A., Izzo, J., . . . Solomon, S. D. (2013). Effect of antihypertensive therapy on ventricular-arterial mechanics, coupling, and efficiency. *Eur Heart J, 34*(9), 676-683. doi: 10.1093/eurheartj/ehs299

Lamba, S., & Abraham, W. T. (2000). Alterations in adrenergic receptor signaling in heart failure. *Heart Fail Rev, 5*(1), 7-16. doi: 10.1023/a:1009885822076

Lammerding, J., Kamm, R. D., & Lee, R. T. (2004). Mechanotransduction in cardiac myocytes. *Ann N Y Acad Sci, 1015*, 53-70. doi: 10.1196/annals.1302.005

Lang, R. M., Bierig, M., Devereux, R. B., Flachskampf, F. A., Foster, E., Pellikka, P. A., . . . Stewart, W. J. (2005). Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *J Am Soc Echocardiogr, 18*(12), 1440-1463. doi: 10.1016/j.echo.2005.10.005

Larstorp, A. C., Okin, P. M., Devereux, R. B., Olsen, M. H., Ibsen, H., Dahlof, B., . . . Wachtell, K. (2012). Regression of ECG-LVH is associated with lower risk of new-onset heart failure and mortality in patients with isolated systolic hypertension; The LIFE study. *Am J Hypertens, 25*(10), 1101-1109. doi: 10.1038/ajh.2012.86

Layland, J., Li, J. M., & Shah, A. M. (2002). Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes. *J Physiol, 540*(Pt 2), 457-467.

Lee, B. H., & Ruoslahti, E. (2005). alpha5beta1 integrin stimulates Bcl-2 expression and cell survival through Akt, focal adhesion kinase, and Ca2+/calmodulin-dependent protein kinase IV. *J Cell Biochem, 95*(6), 1214-1223. doi: 10.1002/jcb.20488

Lee, J. W., & Juliano, R. L. (2000). alpha5beta1 integrin protects intestinal epithelial cells from apoptosis through a phosphatidylinositol 3-kinase and protein kinase B-dependent pathway. *Mol Biol Cell, 11*(6), 1973-1987.

Leenen, F. H., Burns, R. J., Myers, M. G., & Frankel, D. (1990). Effects of nifedipine versus hydralazine on sympathetic activity and cardiac function in patients with hypertension persisting on diuretic plus beta-blocker therapy. *Cardiovasc Drugs Ther, 4*(2), 499-504.

Leicht, M., Briest, W., & Zimmer, H. G. (2003). Regulation of norepinephrine-induced proliferation in cardiac fibroblasts by interleukin-6 and p42/p44 mitogen activated protein kinase. *Mol Cell Biochem, 243*(1-2), 65-72.

Leicht, M., Greipel, N., & Zimmer, H. (2000). Comitogenic effect of catecholamines on rat cardiac fibroblasts in culture. *Cardiovasc Res, 48*(2), 274-284.

Levrand, S., Vannay-Bouchiche, C., Pesse, B., Pacher, P., Feihl, F., Waeber, B., & Liaudet, L. (2006). Peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo. *Free Radic Biol Med, 41*(6), 886-895. doi: 10.1016/j.freeradbiomed.2006.04.034

Levy, D., Kenchaiah, S., Larson, M. G., Benjamin, E. J., Kupka, M. J., Ho, K. K., . . . Vasan, R. S. (2002). Long-term trends in the incidence of and survival with heart failure. *N Engl J Med, 347*(18), 1397-1402. doi: 10.1056/NEJMoa020265

Levy, M. N., Koeppen, B. M., & Stanton, B. A. (2006). *Levy: Berne and Levy's Principles of Physiology*. (4$^{e}$ éd.).

Li, D., Wen, J. F., Jin, J. Y., Jin, H., Ann, H. S., Kim, S. Z., . . . Cho, K. W. (2003). Histamine inhibits atrial myocytic ANP release via H2 receptor-cAMP-protein kinase signaling. *Am J Physiol Regul Integr Comp Physiol, 285*(2), R380-393. doi: 10.1152/ajpregu.00666.2002

Li, Y., Sarkar, O., Brochu, M., & Anand-Srivastava, M. B. (2014). Natriuretic Peptide receptor-C attenuates hypertension in spontaneously hypertensive rats: role of nitroxidative stress and gi proteins. *Hypertension, 63*(4), 846-855. doi: 10.1161/hypertensionaha.113.01772

Li, Z., Bing, O. H., Long, X., Robinson, K. G., & Lakatta, E. G. (1997). Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat. *Am J Physiol, 272*(5 Pt 2), H2313-2319.

Liang, H., Muller, J., Weng, Y. G., Wallukat, G., Fu, P., Lin, H. S., . . . Hetzer, R. (2004). Changes in myocardial collagen content before and after left ventricular assist device application in dilated cardiomyopathy. *Chin Med J (Engl), 117*(3), 401-407.

Liao, Y., Asakura, M., Takashima, S., Ogai, A., Asano, Y., Shintani, Y., . . . Kitakaze, M. (2004). Celiprolol, a vasodilatory beta-blocker, inhibits pressure overload-induced cardiac hypertrophy and prevents the transition to heart failure via nitric oxide-dependent mechanisms in mice. *Circulation, 110*(6), 692-699. doi: 10.1161/01.cir.0000137831.08683.e1

Lijnen, P. J., Petrov, V. V., & Fagard, R. H. (2000). Induction of cardiac fibrosis by angiotensin II. *Methods Find Exp Clin Pharmacol, 22*(10), 709-723.

Lim, K. P., & Hong, W. (2004). Human Nischarin/imidazoline receptor antisera-selected protein is targeted to the endosomes by a combined action of a PX domain and a coiled-coil region. *J Biol Chem, 279*(52), 54770-54782. doi: 10.1074/jbc.M411315200

Lin, X., Hanze, J., Heese, F., Sodmann, R., & Lang, R. E. (1995). Gene expression of natriuretic peptide receptors in myocardial cells. *Circ Res, 77*(4), 750-758.

Lin, Y. H., Wu, X. M., Lee, H. H., Lee, J. K., Liu, Y. C., Chang, H. W., . . . Wu, K. D. (2012). Adrenalectomy reverses myocardial fibrosis in patients with primary aldosteronism. *J Hypertens, 30*(8), 1606-1613. doi: 10.1097/HJH.0b013e3283550f93

Liu, M., Skinner, S. J., Xu, J., Han, R. N., Tanswell, A. K., & Post, M. (1992). Stimulation of fetal rat lung cell proliferation in vitro by mechanical stretch. *Am J Physiol, 263*(3 Pt 1), L376-383.

Lo, M., Liu, K. L., Lantelme, P., & Sassard, J. (1995). Subtype 2 of angiotensin II receptors controls pressure-natriuresis in rats. *J Clin Invest, 95*(3), 1394-1397. doi: 10.1172/jci117792

Long, C. S. (2001). The role of interleukin-1 in the failing heart. *Heart Fail Rev, 6*(2), 81-94.

Lopez, M. J., Wong, S. K., Kishimoto, I., Dubois, S., Mach, V., Friesen, J., . . . Beuve, A. (1995). Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for atrial natriuretic peptide. *Nature, 378*(6552), 65-68. doi: 10.1038/378065a0

Louch, W. E., Sheehan, K. A., & Wolska, B. M. (2011). Methods in cardiomyocyte isolation, culture, and gene transfer. *J Mol Cell Cardiol, 51*(3), 288-298. doi: 10.1016/j.yjmcc.2011.06.012

Lu, Y., Zhou, J., Xu, C., Lin, H., Xiao, J., Wang, Z., & Yang, B. (2008). JAK/STAT and PI3K/AKT pathways form a mutual transactivation loop and afford resistance to oxidative stress-induced apoptosis in cardiomyocytes. *Cell Physiol Biochem, 21*(4), 305-314. doi: 10.1159/000129389

Maack, C., Kartes, T., Kilter, H., Schafers, H. J., Nickenig, G., Bohm, M., & Laufs, U. (2003). Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. *Circulation, 108*(13), 1567-1574. doi: 10.1161/01.cir.0000091084.46500.bb

Machida, T., Hamaya, Y., Izumi, S., Hamaya, Y., Iizuka, K., Igarashi, Y., . . . Hirafuji, M. (2008). Sphingosine 1-phosphate inhibits nitric oxide production induced by interleukin-1beta in rat vascular smooth muscle cells. *J. Pharmacol. Exp. Ther., 325*(1), 200-209. doi: 10.1124/jpet.107.127290

Malhotra, R., & Brosius, F. C., 3rd. (1999). Glucose uptake and glycolysis reduce hypoxia-induced apoptosis in cultured neonatal rat cardiac myocytes. *J Biol Chem, 274*(18), 12567-12575.

Mall, G., Greber, D., Gharenhbaghi, H., Wiest, G., Amann, K., & Mattfeldt, T. (1991). Effects of nifedipine and moxonidine on cardiac structure in spontaneously hypertensive rats (SHR)--stereological studies on myocytes, capillaries, arteries, and cardiac interstitium. *Basic Res Cardiol, 86 Suppl 3*, 33-44.

Mancia, G., De Backer, G., Dominiczak, A., Cifkova, R., Fagard, R., Germano, G., . . . Williams, B. (2007). 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens, 25*(6), 1105-1187. doi: 10.1097/HJH.0b013e3281fc975a

Mann, D. L., McMurray, J. J., Packer, M., Swedberg, K., Borer, J. S., Colucci, W. S., . . . Fleming, T. (2004). Targeted anticytokine therapy in patients with chronic heart failure: results of the Randomized Etanercept Worldwide Evaluation (RENEWAL). *Circulation, 109*(13), 1594-1602. doi: 10.1161/01.cir.0000124490.27666.b2

Mann, D. L., & Young, J. B. (1994). Basic mechanisms in congestive heart failure. Recognizing the role of proinflammatory cytokines. *Chest, 105*(3), 897-904.

Manrique, C., Lastra, G., Gardner, M., & Sowers, J. R. (2009). The renin angiotensin aldosterone system in hypertension: roles of insulin resistance and oxidative stress. *Med Clin North Am, 93*(3), 569-582. doi: 10.1016/j.mcna.2009.02.014

Martin, U., Hill, C., & O'Mahony, D. (2005). Use of moxonidine in elderly patients with resistant hypertension. *J Clin Pharm Ther, 30*(5), 433-437. doi: 10.1111/j.1365-2710.2005.00672.x

Mathers, C. D., & Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med, 3*(11), e442. doi: 10.1371/journal.pmed.0030442

McGrath, M. F., & de Bold, A. J. (2005). Determinants of natriuretic peptide gene expression. *Peptides, 26*(6), 933-943. doi: 10.1016/j.peptides.2004.12.022

Means, C. K., & Brown, J. H. (2009). Sphingosine-1-phosphate receptor signalling in the heart. *Cardiovasc Res, 82*(2), 193-200. doi: 10.1093/cvr/cvp086

Melo, L. G., Steinhelper, M. E., Pang, S. C., Tse, Y., & Ackermann, U. (2000). ANP in regulation of arterial pressure and fluid-electrolyte balance: lessons from genetic mouse models. *Physiol Genomics, 3*(1), 45-58.

Melo, L. G., Veress, A. T., Ackermann, U., Pang, S. C., Flynn, T. G., & Sonnenberg, H. (1999). Chronic hypertension in ANP knockout mice: contribution of peripheral resistance. *Regul Pept, 79*(2-3), 109-115.

Menaouar, A., El-Ayoubi, R., Jankowski, M., Gutkowska, J., & Mukaddam-Daher, S. (2002). Chronic imidazoline receptor activation in spontaneously hypertensive rats. *Am J Hypertens, 15*(9), 803-808.

Meredith, I. T., Eisenhofer, G., Lambert, G. W., Dewar, E. M., Jennings, G. L., & Esler, M. D. (1993). Cardiac sympathetic nervous activity in congestive heart failure. Evidence for increased neuronal norepinephrine release and preserved neuronal uptake. *Circulation, 88*(1), 136-145.

Meszaros, J. G., Gonzalez, A. M., Endo-Mochizuki, Y., Villegas, S., Villarreal, F., & Brunton, L. L. (2000). Identification of G protein-coupled signaling pathways in cardiac fibroblasts: cross talk between G(q) and G(s). *Am J Physiol Cell Physiol, 278*(1), C154-162.

Metcalfe, B. L., Huentelman, M. J., Parilak, L. D., Taylor, D. G., Katovich, M. J., Knot, H. J., . . . Raizada, M. K. (2004). Prevention of cardiac hypertrophy by angiotensin II type-2 receptor gene transfer. *Hypertension, 43*(6), 1233-1238. doi: 10.1161/01.hyp.0000127563.14064.fd

Mittal, A., Pulina, M., Hou, S. Y., & Astrof, S. (2010). Fibronectin and integrin alpha 5 play essential roles in the development of the cardiac neural crest. *Mech Dev, 127*(9-12), 472-484. doi: 10.1016/j.mod.2010.08.005

Mittal, A., Pulina, M., Hou, S. Y., & Astrof, S. (2013). Fibronectin and integrin alpha 5 play requisite roles in cardiac morphogenesis. *Dev Biol, 381*(1), 73-82. doi: 10.1016/j.ydbio.2013.06.010

Mizote, I., Yamaguchi, O., Hikoso, S., Takeda, T., Taneike, M., Oka, T., . . . Otsu, K. (2010). Activation of MTK1/MEKK4 induces cardiomyocyte death and heart failure. *J Mol Cell Cardiol, 48*(2), 302-309. doi: 10.1016/j.yjmcc.2009.10.010

Mobini, R., Fu, M., Jansson, P. A., Bergh, C. H., Scharin Tang, M., Waagstein, F., & Andersson, B. (2006). Influence of central inhibition of sympathetic nervous activity on myocardial metabolism in chronic heart failure: acute effects of the imidazoline I1-receptor agonist moxonidine. *Clin Sci (Lond), 110*(3), 329-336. doi: 10.1042/cs20050037

Moga, M. A., Nakamura, T., & Robbins, J. (2008). Genetic approaches for changing the heart and dissecting complex syndromes. *J Mol Cell Cardiol, 45*(2), 148-155. doi: 10.1016/j.yjmcc.2008.06.002

Molderings, G. J., Bonisch, H., Bruss, M., Wolf, C., von Kugelgen, I., & Gothert, M. (2007). S1P-receptors in PC12 and transfected HEK293 cells: molecular targets of hypotensive imidazoline I(1) receptor ligands. *Neurochem Int, 51*(8), 476-485. doi: 10.1016/j.neuint.2007.04.022

Molderings, G. J., Bonisch, H., Hammermann, R., Gothert, M., & Bruss, M. (2002). Noradrenaline release-inhibiting receptors on PC12 cells devoid of alpha(2(-)) and CB(1) receptors: similarities to presynaptic imidazoline and edg receptors. *Neurochem Int, 40*(2), 157-167.

Molderings, G. J., & Gothert, M. (1998). Presynaptic imidazoline receptors mediate inhibition of noradrenaline release from sympathetic nerves in rat blood vessels. *Fundam Clin Pharmacol, 12*(4), 388-397.

Moore, J. E., Jr., Burki, E., Suciu, A., Zhao, S., Burnier, M., Brunner, H. R., & Meister, J. J. (1994). A device for subjecting vascular endothelial cells to both fluid shear stress and circumferential cyclic stretch. *Ann Biomed Eng, 22*(4), 416-422.

Morgan, N. G., & Chan, S. L. (2001). Imidazoline binding sites in the endocrine pancreas: can they fulfil their potential as targets for the development of new insulin secretagogues? *Curr Pharm Des, 7*(14), 1413-1431.

Morisco, C., Zebrowski, D. C., Vatner, D. E., Vatner, S. F., & Sadoshima, J. (2001). Beta-adrenergic cardiac hypertrophy is mediated primarily by the beta(1)-subtype in the rat heart. *J Mol Cell Cardiol, 33*(3), 561-573. doi: 10.1006/jmcc.2000.1332

Mukaddam-Daher, S. (2006). Natriuretic peptides as therapeutic targets. *Expert Opin Ther Targets, 10*(2), 239-252. doi: 10.1517/14728222.10.2.239

Mukaddam-Daher, S., & Gutkowska, J. (1999). The renal actions of moxonidine are mediated by atrial natriuretic peptide and involve the opioid receptors. *Ann N Y Acad Sci, 881*, 385-387.

Mukaddam-Daher, S., & Gutkowska, J. (2000). Atrial natriuretic peptide is involved in renal actions of moxonidine. *Hypertension, 35*(6), 1215-1220.

Mukaddam-Daher, S., Lambert, C., & Gutkowska, J. (1997). Clonidine and ST-91 may activate imidazoline binding sites in the heart to release atrial natriuretic peptide. *Hypertension, 30*(1 Pt 1), 83-87.

Mukaddam-Daher, S., Menaouar, A., & Gutkowska, J. (2006). Receptors involved in moxonidine-stimulated atrial natriuretic peptide release from isolated normotensive rat hearts. *Eur J Pharmacol, 541*(1-2), 73-79. doi: 10.1016/j.ejphar.2006.05.009

Mukaddam-Daher, S., Menaouar, A., Paquette, P. A., Jankowski, M., Gutkowska, J., Gillis, M. A., . . . Tardif, J. C. (2009). Hemodynamic and cardiac effects of chronic eprosartan

and moxonidine therapy in stroke-prone spontaneously hypertensive rats. *Hypertension, 53*(5), 775-781. doi: 10.1161/HYPERTENSIONAHA.108.126524

Murthy, K. S., Teng, B., Jin, J., & Makhlouf, G. M. (1998). G protein-dependent activation of smooth muscle eNOS via natriuretic peptide clearance receptor. *Am J Physiol, 275*(6 Pt 1), C1409-1416.

Nakamura, S., Naruse, M., Naruse, K., Kawana, M., Nishikawa, T., Hosoda, S., . . . et al. (1991). Atrial natriuretic peptide and brain natriuretic peptide coexist in the secretory granules of human cardiac myocytes. *Am J Hypertens, 4*(11), 909-912.

Nap, A., Balt, J. C., Mathy, M. J., & Van Zwieten, P. A. (2003). AT(1)-receptor blockade and sympathetic neurotransmission in cardiovascular disease. *Auton Autacoid Pharmacol, 23*(5-6), 285-296. doi: 10.1111/j.1474-8673.2004.00301.x

Nawata, J., Ohno, I., Isoyama, S., Suzuki, J., Miura, S., Ikeda, J., & Shirato, K. (1999). Differential expression of alpha 1, alpha 3 and alpha 5 integrin subunits in acute and chronic stages of myocardial infarction in rats. *Cardiovasc Res, 43*(2), 371-381.

Neumann, J., Ligtenberg, G., Oey, L., Koomans, H. A., & Blankestijn, P. J. (2004). Moxonidine normalizes sympathetic hyperactivity in patients with eprosartan-treated chronic renal failure. *J Am Soc Nephrol, 15*(11), 2902-2907. doi: 10.1097/01.asn.0000143471.10750.8c

Nguyen Dinh Cat, A., & Touyz, R. M. (2011). A new look at the renin-angiotensin system--focusing on the vascular system. *Peptides, 32*(10), 2141-2150. doi: 10.1016/j.peptides.2011.09.010

Nielsen, I. (1986). The natural history of hypertensive heart disease as suggested by echocardiography. *Acta Med Scand Suppl, 714*, 165-169.

Nolly, M. B., Caldiz, C. I., Yeves, A. M., Villa-Abrille, M. C., Morgan, P. E., Amado Mondaca, N., . . . Ennis, I. L. (2014). The signaling pathway for aldosterone-induced mitochondrial production of superoxide anion in the myocardium. *J Mol Cell Cardiol, 67*, 60-68. doi: 10.1016/j.yjmcc.2013.12.004

Ogawa, T., & de Bold, A. J. (2014). The heart as an endocrine organ. *Endocr Connect, 3*(2), R31-44. doi: 10.1530/EC-14-0012

Ogawa, T., Vatta, M., Bruneau, B. G., & de Bold, A. J. (1999). Characterization of natriuretic peptide production by adult heart atria. *Am J Physiol, 276*(6 Pt 2), H1977-1986.

Ohno, M., Takemura, G., Ohno, A., Misao, J., Hayakawa, Y., Minatoguchi, S., . . . Fujiwara, H. (1998). "Apoptotic" myocytes in infarct area in rabbit hearts may be oncotic myocytes with DNA fragmentation: analysis by immunogold electron microscopy combined with In situ nick end-labeling. *Circulation, 98*(14), 1422-1430.

Okamoto, K., & Aoki, K. (1963). Development of a strain of spontaneously hypertensive rats. *Jpn Circ J, 27*, 282-293.

Ollivier, J. P., & Christen, M. O. (1994). I1-imidazoline-receptor agonists in the treatment of hypertension: an appraisal of clinical experience. *J Cardiovasc Pharmacol, 24 Suppl 1*, S39-48.

Orogo, A. M., & Gustafsson, A. B. (2013). Cell death in the myocardium: my heart won't go on. *IUBMB Life, 65*(8), 651-656. doi: 10.1002/iub.1180

Osadchii, O. E. (2007). Cardiac hypertrophy induced by sustained beta-adrenoreceptor activation: pathophysiological aspects. *Heart Fail Rev, 12*(1), 66-86. doi: 10.1007/s10741-007-9007-4

Packer, M., Bristow, M. R., Cohn, J. N., Colucci, W. S., Fowler, M. B., Gilbert, E. M., & Shusterman, N. H. (1996). The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group. *N Engl J Med, 334*(21), 1349-1355. doi: 10.1056/nejm199605233342101

Palatini, P., Frigo, G., Visentin, P., Mario, L., Mormino, P., & Pessina, A. C. (2001). Left ventricular contractile performance in the early stage of hypertension in humans. *Eur J Appl Physiol, 85*(1-2), 118-124.

Pandey, K. N., Nguyen, H. T., Li, M., & Boyle, J. W. (2000). Natriuretic peptide receptor-A negatively regulates mitogen-activated protein kinase and proliferation of mesangial cells: role of cGMP-dependent protein kinase. *Biochem Biophys Res Commun, 271*(2), 374-379. doi: 10.1006/bbrc.2000.2627

Pappachan, J. M., Varughese, G. I., Sriraman, R., & Arunagirinathan, G. (2013). Diabetic cardiomyopathy: Pathophysiology, diagnostic evaluation and management. *World J Diabetes, 4*(5), 177-189. doi: 10.4239/wjd.v4.i5.177

Paquette, P. A., Duguay, D., El-Ayoubi, R., Menaouar, A., Danalache, B., Gutkowska, J., . . . Mukaddam-Daher, S. (2008). Control of left ventricular mass by moxonidine involves reduced DNA synthesis and enhanced DNA fragmentation. *Br J Pharmacol, 153*(3), 459-467. doi: 10.1038/sj.bjp.0707588

Parati, G., & Esler, M. (2012). The human sympathetic nervous system: its relevance in hypertension and heart failure. *Eur Heart J, 33*(9), 1058-1066. doi: 10.1093/eurheartj/ehs041

Peiro, C., Vallejo, S., Gembardt, F., Palacios, E., Novella, S., Azcutia, V., . . . Walther, T. (2013). Complete blockade of the vasorelaxant effects of angiotensin-(1-7) and bradykinin in murine microvessels by antagonists of the receptor Mas. *J Physiol, 591*(Pt 9), 2275-2285. doi: 10.1113/jphysiol.2013.251413

Pelliccia, A., Maron, B. J., Spataro, A., Proschan, M. A., & Spirito, P. (1991). The upper limit of physiologic cardiac hypertrophy in highly trained elite athletes. *N Engl J Med, 324*(5), 295-301. doi: 10.1056/nejm199101313240504

Peng, H. J., Dai, D. Z., Ji, H., & Dai, Y. (2010). The separate roles of endothelin receptors participate in remodeling of matrix metalloproteinase and connexin 43 of cardiac fibroblasts in maladaptive response to isoproterenol. *Eur J Pharmacol, 634*(1-3), 101-106. doi: 10.1016/j.ejphar.2010.02.001

Perlini, S., Ferrero, I., Palladini, G., Tozzi, R., Gatti, C., Vezzoli, M., . . . Ferrari, A. U. (2006). Survival benefits of different antiadrenergic interventions in pressure overload left ventricular hypertrophy/failure. *Hypertension, 48*(1), 93-97. doi: 10.1161/01.HYP.0000226859.42064.ea

Petrov, V. V., Fagard, R. H., & Lijnen, P. J. (2002). Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension, 39*(2), 258-263.

Piao, F. L., Cao, C., Han, J. H., Kim, S. Z., & Kim, S. H. (2004). Calcitonin gene-related peptide-induced suppression of atrial natriuretic peptide release through receptors for CGRP1 but not for calcitonin and amylin. *Eur J Pharmacol, 483*(2-3), 295-300.

Picatoste, B., Ramirez, E., Caro-Vadillo, A., Iborra, C., Ares-Carrasco, S., Egido, J., . . . Lorenzo, O. (2013). Sitagliptin reduces cardiac apoptosis, hypertrophy and fibrosis primarily by insulin-dependent mechanisms in experimental type-II diabetes. Potential

roles of GLP-1 isoforms. *PLoS One, 8*(10), e78330. doi: 10.1371/journal.pone.0078330

Piletz, J. E., Ivanov, T. R., Sharp, J. D., Ernsberger, P., Chang, C. H., Pickard, R. T., . . . Reis, D. J. (2000). Imidazoline receptor antisera-selected (IRAS) cDNA: cloning and characterization. *DNA Cell Biol, 19*(6), 319-329. doi: 10.1089/10445490050043290

Piletz, J. E., Jones, J. C., Zhu, H., Bishara, O., & Ernsberger, P. (1999). Imidazoline receptor antisera-selected cDNA clone and mRNA distribution. *Ann N Y Acad Sci, 881*, 1-7.

Pinheiro, S. V., Ferreira, A. J., Kitten, G. T., da Silveira, K. D., da Silva, D. A., Santos, S. H., . . . Simoes e Silva, A. C. (2009). Genetic deletion of the angiotensin-(1-7) receptor Mas leads to glomerular hyperfiltration and microalbuminuria. *Kidney Int, 75*(11), 1184-1193. doi: 10.1038/ki.2009.61

Pischon, T., & Sharma, A. M. (2002). Recent developments in the treatment of obesity-related hypertension. *Curr Opin Nephrol Hypertens, 11*(5), 497-502.

Planitz, V. (1987). Comparison of moxonidine and clonidine HCl in treating patients with hypertension. *J Clin Pharmacol, 27*(1), 46-51.

Poirier, L., & Lacourciere, Y. (2012). The evolving role of beta-adrenergic receptor blockers in managing hypertension. *Can J Cardiol, 28*(3), 334-340. doi: 10.1016/j.cjca.2012.04.001

Porter, K. E., & Turner, N. A. (2009). Cardiac fibroblasts: at the heart of myocardial remodeling. *Pharmacol Ther, 123*(2), 255-278. doi: 10.1016/j.pharmthera.2009.05.002

Poyhonen-Alho, M. K., Manhem, K., Katzman, P., Kibarskis, A., Antikainen, R. L., Erkkola, R. U., . . . Kaaja, R. J. (2008). Central sympatholytic therapy has anti-inflammatory properties in hypertensive postmenopausal women. *J Hypertens, 26*(12), 2445-2449. doi: 10.1097/HJH.0b013e328311cf37

Prichard, B. N., Jager, B. A., Luszick, J. H., Kuster, L. J., Verboom, C. N., Hughes, P. R., . . . Kuppers, H. E. (2002). Placebo-controlled comparison of the efficacy and tolerability of once-daily moxonidine and enalapril in mild to moderate essential hypertension. *Blood Press, 11*(3), 166-172.

Prins, B. A., Weber, M. J., Hu, R. M., Pedram, A., Daniels, M., & Levin, E. R. (1996). Atrial natriuretic peptide inhibits mitogen-activated protein kinase through the clearance receptor. Potential role in the inhibition of astrocyte proliferation. *J Biol Chem, 271*(24), 14156-14162.

Pruthi, D., McCurley, A., Aronovitz, M., Galayda, C., Karumanchi, S. A., & Jaffe, I. Z. (2014). Aldosterone promotes vascular remodeling by direct effects on smooth muscle cell mineralocorticoid receptors. *Arterioscler Thromb Vasc Biol, 34*(2), 355-364. doi: 10.1161/atvbaha.113.302854

Raasch, W., Jungbluth, B., Schafer, U., Hauser, W., & Dominiak, P. (2003). Norepinephrine release is reduced by I(1)-receptors in addition to alpha(2)-adrenoceptors. *Ann N Y Acad Sci, 1009*, 270-273.

Razavi, H. M., Hamilton, J. A., & Feng, Q. (2005). Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. *Pharmacol Ther, 106*(2), 147-162. doi: 10.1016/j.pharmthera.2004.11.006

Remick, D. G., Call, D. R., Ebong, S. J., Newcomb, D. E., Nybom, P., Nemzek, J. A., & Bolgos, G. E. (2001). Combination immunotherapy with soluble tumor necrosis factor receptors plus interleukin 1 receptor antagonist decreases sepsis mortality. *Crit Care Med, 29*(3), 473-481.

Reusch, P., Wagdy, H., Reusch, R., Wilson, E., & Ives, H. E. (1996). Mechanical strain increases smooth muscle and decreases nonmuscle myosin expression in rat vascular smooth muscle cells. *Circ Res, 79*(5), 1046-1053.

Robitaille, C., Dai, S., Waters, C., Loukine, L., Bancej, C., Quach, S., . . . Quan, H. (2012). Diagnosed hypertension in Canada: incidence, prevalence and associated mortality. *Cmaj, 184*(1), E49-56. doi: 10.1503/cmaj.101863

Roger, V. L., Weston, S. A., Redfield, M. M., Hellermann-Homan, J. P., Killian, J., Yawn, B. P., & Jacobsen, S. J. (2004). Trends in heart failure incidence and survival in a community-based population. *Jama, 292*(3), 344-350. doi: 10.1001/jama.292.3.344

Rohr, S. (2011). Cardiac fibroblasts in cell culture systems: myofibroblasts all along? *J Cardiovasc Pharmacol, 57*(4), 389-399. doi: 10.1097/FJC.0b013e3182137e17

Ross, R. S. (2002). The extracellular connections: the role of integrins in myocardial remodeling. *J Card Fail, 8*(6 Suppl), S326-331. doi: 10.1054/jcaf.2002.129263

Ross, R. S., & Borg, T. K. (2001). Integrins and the myocardium. *Circ Res, 88*(11), 1112-1119.

Ross, R. S., Pham, C., Shai, S. Y., Goldhaber, J. I., Fenczik, C., Glembotski, C. C., . . . Loftus, J. C. (1998). Beta1 integrins participate in the hypertrophic response of rat ventricular myocytes. *Circ Res, 82*(11), 1160-1172.

Ross, T. D., Coon, B. G., Yun, S., Baeyens, N., Tanaka, K., Ouyang, M., & Schwartz, M. A. (2013). Integrins in mechanotransduction. *Curr Opin Cell Biol, 25*(5), 613-618. doi: 10.1016/j.ceb.2013.05.006

Rossier, M. F., Python, M., & Maturana, A. D. (2010). Contribution of mineralocorticoid and glucocorticoid receptors to the chronotropic and hypertrophic actions of aldosterone in neonatal rat ventricular myocytes. *Endocrinology, 151*(6), 2777-2787. doi: 10.1210/en.2009-1375

Ruoslahti, E. (1991). Integrins. *J Clin Invest, 87*(1), 1-5. doi: 10.1172/jci114957

Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T. J., & Izumo, S. (1992). Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J Biol Chem, 267*(15), 10551-10560.

Sakane, K. K., Monteiro, C. J., Silva, W., Silva, A. R., Santos, P. M., Lima, K. F., & Moraes, K. C. (2014). Cellular and molecular studies of the effects of a selective COX-2 inhibitor celecoxib in the cardiac cell line H9c2 and their correlation with death mechanisms. *Braz J Med Biol Res, 47*(1), 50-59. doi: 10.1590/1414-431x20133028

Sakurai, T., Yanagisawa, M., Inoue, A., Ryan, U. S., Kimura, S., Mitsui, Y., . . . Masaki, T. (1991). cDNA cloning, sequence analysis and tissue distribution of rat preproendothelin-1 mRNA. *Biochem Biophys Res Commun, 175*(1), 44-47.

Sanjuliani, A. F., Francischetti, E. A., Genelhu de Abreu, V., & Ueleres Braga, J. (2004). Effects of Moxonidine on the Sympathetic Nervous System, Blood Pressure, Plasma Renin Activity, Plasma Aldosterone, Leptin, and Metabolic Profile in Obese Hypertensive Patients. *Journal of clinical and basic cardiology, 7*(1-4), 19-25.

Sano, H., Liu, S. C., Lane, W. S., Piletz, J. E., & Lienhard, G. E. (2002). Insulin receptor substrate 4 associates with the protein IRAS. *J Biol Chem, 277*(22), 19439-19447. doi: 10.1074/jbc.M111838200

Santiago, J. J., Dangerfield, A. L., Rattan, S. G., Bathe, K. L., Cunnington, R. H., Raizman, J. E., . . . Dixon, I. M. (2010). Cardiac fibroblast to myofibroblast differentiation in vivo

and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. *Dev Dyn, 239*(6), 1573-1584. doi: 10.1002/dvdy.22280

Santos, R. A. (2014). Angiotensin-(1-7). *Hypertension, 63*(6), 1138-1147. doi: 10.1161/hypertensionaha.113.01274

Santos, R. A., Simoes e Silva, A. C., Maric, C., Silva, D. M., Machado, R. P., de Buhr, I., . . . Walther, T. (2003). Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A, 100*(14), 8258-8263. doi: 10.1073/pnas.1432869100

Satia, M. C., Damani, R. R., & Goyal, R. K. (1997). Beneficial effects of clonidine in streptozotocin-induced diabetes and DOCA-hypertensive rats. *J Pharm Pharmacol, 49*(10), 1030-1035.

Satoh, M., Ogita, H., Takeshita, K., Mukai, Y., Kwiatkowski, D. J., & Liao, J. K. (2006). Requirement of Rac1 in the development of cardiac hypertrophy. *Proc Natl Acad Sci U S A, 103*(19), 7432-7437. doi: 10.1073/pnas.0510444103

Schafer, U., Burgdorf, C., Engelhardt, A., Raasch, W., Kurz, T., & Richardt, G. (2003). Moxonidine displays a presynaptic alpha-2-adrenoceptor-dependent synergistic sympathoinhibitory action at imidazoline-1 receptors. *Ann N Y Acad Sci, 1009*, 265-269.

Schiavone, M. T., Santos, R. A., Brosnihan, K. B., Khosla, M. C., & Ferrario, C. M. (1988). Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide. *Proc Natl Acad Sci U S A, 85*(11), 4095-4098.

Schiebinger, R. J., & Greening, K. M. (1992). Interaction between stretch and hormonally stimulated atrial natriuretic peptide secretion. *Am J Physiol, 262*(1 Pt 2), H78-83.

Schiffrin, E. L. (2013). The immune system: role in hypertension. *Can J Cardiol, 29*(5), 543-548. doi: 10.1016/j.cjca.2012.06.009

Schilling, J. D., & Mann, D. L. (2012). Diabetic cardiomyopathy: bench to bedside. *Heart Fail Clin, 8*(4), 619-631. doi: 10.1016/j.hfc.2012.06.007

Schlaich, M. P., Kaye, D. M., Lambert, E., Sommerville, M., Socratous, F., & Esler, M. D. (2003). Relation between cardiac sympathetic activity and hypertensive left ventricular hypertrophy. *Circulation, 108*(5), 560-565. doi: 10.1161/01.cir.0000081775.72651.b6

Seeland, U., Selejan, S., Engelhardt, S., Muller, P., Lohse, M. J., & Bohm, M. (2007). Interstitial remodeling in beta1-adrenergic receptor transgenic mice. *Basic Res Cardiol, 102*(2), 183-193. doi: 10.1007/s00395-006-0635-y

Separovic, D., Kester, M., & Ernsberger, P. (1996). Coupling of I1-imidazoline receptors to diacylglyceride accumulation in PC12 rat pheochromocytoma cells. *Mol Pharmacol, 49*(4), 668-675.

Separovic, D., Kester, M., Haxhiu, M. A., & Ernsberger, P. (1997). Activation of phosphatidylcholine-selective phospholipase C by I1-imidazoline receptors in PC12 cells and rostral ventrolateral medulla. *Brain Res, 749*(2), 335-339. doi: 10.1016/s0006-8993(96)01372-8

Shannon, R., & Chaudhry, M. (2006). Effect of alpha1-adrenergic receptors in cardiac pathophysiology. *Am Heart J, 152*(5), 842-850. doi: 10.1016/j.ahj.2006.05.017

Shapiro, B. A., Olala, L., Arun, S. N., Parker, P. M., George, M. V., & Bollag, W. B. (2010). Angiotensin II-activated protein kinase D mediates acute aldosterone secretion. *Mol Cell Endocrinol, 317*(1-2), 99-105. doi: 10.1016/j.mce.2009.11.017

Sharma, A. M., Wagner, T., & Marsalek, P. (2004). Moxonidine in the treatment of overweight and obese patients with the metabolic syndrome: a postmarketing surveillance study. *J Hum Hypertens, 18*(9), 669-675. doi: 10.1038/sj.jhh.1001676

Shiels, H. A., & White, E. (2008). The Frank-Starling mechanism in vertebrate cardiac myocytes. *J Exp Biol, 211*(Pt 13), 2005-2013. doi: 10.1242/jeb.003145

Shindo, T., Ikeda, U., Ohkawa, F., Kawahara, Y., Yokoyama, M., & Shimada, K. (1995). Nitric oxide synthesis in cardiac myocytes and fibroblasts by inflammatory cytokines. *Cardiovasc Res, 29*(6), 813-819.

Shioi, T., Matsumori, A., Kihara, Y., Inoko, M., Ono, K., Iwanaga, Y., . . . Sasayama, S. (1997). Increased expression of interleukin-1 beta and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the hypertrophied and failing heart with pressure overload. *Circ Res, 81*(5), 664-671.

Shyu, K. G. (2009). Cellular and molecular effects of mechanical stretch on vascular cells and cardiac myocytes. *Clin Sci (Lond), 116*(5), 377-389. doi: 10.1042/cs20080163

Silberman, G. A., Fan, T. H., Liu, H., Jiao, Z., Xiao, H. D., Lovelock, J. D., . . . Dudley, S. C., Jr. (2010). Uncoupled cardiac nitric oxide synthase mediates diastolic dysfunction. *Circulation, 121*(4), 519-528. doi: 10.1161/circulationaha.109.883777

Silverthorn, D. (2013). *Human physiology : an integrated approach*. (6$^{e}$ éd.).

Singh, K., Xiao, L., Remondino, A., Sawyer, D. B., & Colucci, W. S. (2001). Adrenergic regulation of cardiac myocyte apoptosis. *J Cell Physiol, 189*(3), 257-265. doi: 10.1002/jcp.10024

Siragy, H. M., & Carey, R. M. (1997). The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest, 100*(2), 264-269. doi: 10.1172/jci119531

Siwik, D. A., Chang, D. L., & Colucci, W. S. (2000). Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. *Circ Res, 86*(12), 1259-1265.

Slama, M., Ahn, J., Varagic, J., Susic, D., & Frohlich, E. D. (2004). Long-term left ventricular echocardiographic follow-up of SHR and WKY rats: effects of hypertension and age. *Am J Physiol Heart Circ Physiol, 286*(1), H181-185. doi: 10.1152/ajpheart.00642.2003

Slama, M., Susic, D., Varagic, J., & Frohlich, E. D. (2002). Diastolic dysfunction in hypertension. *Curr Opin Cardiol, 17*(4), 368-373.

Sole, M. J., Lo, C. M., Laird, C. W., Sonnenblick, E. H., & Wurtman, R. J. (1975). Norepinephrine turnover in the heart and spleen of the cardiomyopathic Syrian hamster. *Circ Res, 37*(6), 855-862.

Spiegel, S., & Milstien, S. (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol, 4*(5), 397-407. doi: 10.1038/nrm1103

Stabile, A. M., Aceros, H., Stockmeyer, K., Abdel Rahman, A. A., Noiseux, N., & Mukaddam-Daher, S. (2011). Functional and molecular effects of imidazoline receptor activation in heart failure. *Life Sci, 88*(11-12), 493-503. doi: 10.1016/j.lfs.2011.01.008

Statistics Canada, H. S. D. (2012). Mortality, Summary List of Causes 2009. Repéré à http://www.statcan.gc.ca/pub/84f0209x/84f0209x2009000-eng.pdf

Stewart, J. A., Jr., Gardner, J. D., Brower, G. L., & Janicki, J. S. (2014). Temporal changes in integrin-mediated cardiomyocyte adhesion secondary to chronic cardiac volume overload in rats. *Am J Physiol Heart Circ Physiol, 306*(1), H101-108. doi: 10.1152/ajpheart.00541.2013

Strauer, B. E. (1984). Functional dynamics of the left ventricle in hypertensive hypertrophy and failure. *Hypertension, 6*(6 Pt 2), Iii4-12.

Strobeck, J. E., Factor, S. M., Bhan, A., Sole, M., Liew, C. C., Fein, F., & Sonnenblick, E. H. (1979). Hereditary and acquired cardiomyopathies in experimental animals: mechanical, biochemical, and structural features. *Ann N Y Acad Sci, 317*, 59-88.

Sugden, P. H., & Clerk, A. (1998). Cellular mechanisms of cardiac hypertrophy. *J Mol Med (Berl), 76*(11), 725-746.

Sumita Yoshikawa, W., Nakamura, K., Miura, D., Shimizu, J., Hashimoto, K., Kataoka, N., . . . Ito, H. (2013). Increased passive stiffness of cardiomyocytes in the transverse direction and residual actin and myosin cross-bridge formation in hypertrophied rat hearts induced by chronic beta-adrenergic stimulation. *Circ J, 77*(3), 741-748.

Sun, Y., Carretero, O. A., Xu, J., Rhaleb, N. E., Yang, J. J., Pagano, P. J., & Yang, X. P. (2009). Deletion of inducible nitric oxide synthase provides cardioprotection in mice with 2-kidney, 1-clip hypertension. *Hypertension, 53*(1), 49-56. doi: 10.1161/hypertensionaha.108.121822

Sun, Z., Chang, C. H., & Ernsberger, P. (2007). Identification of IRAS/Nischarin as an I1-imidazoline receptor in PC12 rat pheochromocytoma cells. *J Neurochem, 101*(1), 99-108. doi: 10.1111/j.1471-4159.2006.04413.x

Sussman, M. A., Welch, S., Walker, A., Klevitsky, R., Hewett, T. E., Price, R. L., . . . Yager, K. (2000). Altered focal adhesion regulation correlates with cardiomyopathy in mice expressing constitutively active rac1. *J Clin Invest, 105*(7), 875-886. doi: 10.1172/jci8497

Swedberg, K., Bergh, C. H., Dickstein, K., McNay, J., & Steinberg, M. (2000). The effects of moxonidine, a novel imidazoline, on plasma norepinephrine in patients with congestive heart failure. Moxonidine Investigators. *J Am Coll Cardiol, 35*(2), 398-404.

Swedberg, K., Bristow, M. R., Cohn, J. N., Dargie, H., Straub, M., Wiltse, C., & Wright, T. J. (2002). Effects of sustained-release moxonidine, an imidazoline agonist, on plasma norepinephrine in patients with chronic heart failure. *Circulation, 105*(15), 1797-1803.

Takemura, G., Fujiwara, H., Horike, K., Mukoyama, M., Saito, Y., Nakao, K., . . . et al. (1989). Ventricular expression of atrial natriuretic polypeptide and its relations with hemodynamics and histology in dilated human hearts. Immunohistochemical study of the endomyocardial biopsy specimens. *Circulation, 80*(5), 1137-1147.

Tamamori, M., Ito, H., Hiroe, M., Marumo, F., & Hata, R. I. (1997). Stimulation of collagen synthesis in rat cardiac fibroblasts by exposure to hypoxic culture conditions and suppression of the effect by natriuretic peptides. *Cell Biol Int, 21*(3), 175-180. doi: 10.1006/cbir.1997.0130

Tamura, N., Ogawa, Y., Chusho, H., Nakamura, K., Nakao, K., Suda, M., . . . Katsuki, M. (2000). Cardiac fibrosis in mice lacking brain natriuretic peptide. *Proc Natl Acad Sci U S A, 97*(8), 4239-4244. doi: 10.1073/pnas.070371497

Tanabe, A., Naruse, M., Arai, K., Naruse, K., Yoshimoto, T., Seki, T., . . . Demura, H. (1998). Angiotensin II stimulates both aldosterone secretion and DNA synthesis via type 1 but not type 2 receptors in bovine adrenocortical cells. *J Endocrinol Invest, 21*(10), 668-672.

Teran, N., Rodriguez-Iturbe, B., Parra, G., & Gutkowska, J. (1991). Atrial natriuretic peptide levels in brain venous outflow during cardiopulmonary bypass in humans: evidence for extracardiac hormonal production. *J Cardiothorac Vasc Anesth, 5*(4), 343-347.

Terracio, L., Rubin, K., Gullberg, D., Balog, E., Carver, W., Jyring, R., & Borg, T. K. (1991). Expression of collagen binding integrins during cardiac development and hypertrophy. *Circ Res, 68*(3), 734-744.

Thibault, G., Amiri, F., & Garcia, R. (1999). Regulation of natriuretic peptide secretion by the heart. *Annu Rev Physiol, 61*, 193-217. doi: 10.1146/annurev.physiol.61.1.193

Thomas, C. J., & Woods, R. L. (2003). Guanylyl cyclase receptors mediate cardiopulmonary vagal reflex actions of ANP. *Hypertension, 41*(2), 279-285.

Tian, B., Liu, J., Bitterman, P. B., & Bache, R. J. (2002). Mechanisms of cytokine induced NO-mediated cardiac fibroblast apoptosis. *Am J Physiol Heart Circ Physiol, 283*(5), H1958-1967. doi: 10.1152/ajpheart.01070.2001

Timonen, P., Magga, J., Risteli, J., Punnonen, K., Vanninen, E., Turpeinen, A., . . . Peuhkurinen, K. (2008). Cytokines, interstitial collagen and ventricular remodelling in dilated cardiomyopathy. *Int J Cardiol, 124*(3), 293-300. doi: 10.1016/j.ijcard.2007.02.004

Toal, C. B., Meredith, P. A., & Elliott, H. L. (2012). Long-acting dihydropyridine calcium-channel blockers and sympathetic nervous system activity in hypertension: a literature review comparing amlodipine and nifedipine GITS. *Blood Press, 21 Suppl 1*, 3-10. doi: 10.3109/08037051.2012.690615

Tobaldini, E., Nobili, L., Strada, S., Casali, K. R., Braghiroli, A., & Montano, N. (2013). Heart rate variability in normal and pathological sleep. *Front Physiol, 4*, 294. doi: 10.3389/fphys.2013.00294

Toldo, S., Mezzaroma, E., Van Tassell, B. W., Farkas, D., Marchetti, C., Voelkel, N. F., & Abbate, A. (2013). Interleukin-1beta blockade improves cardiac remodelling after myocardial infarction without interrupting the inflammasome in the mouse. *Exp Physiol, 98*(3), 734-745. doi: 10.1113/expphysiol.2012.069831

Tomaschitz, A., Pilz, S., Ritz, E., Obermayer-Pietsch, B., & Pieber, T. R. (2010). Aldosterone and arterial hypertension. *Nat Rev Endocrinol, 6*(2), 83-93. doi: 10.1038/nrendo.2009.263

Trenk, D., Wagner, F., Jahnchen, E., & Planitz, V. (1987). Pharmacokinetics of moxonidine after single and repeated daily doses in healthy volunteers. *J Clin Pharmacol, 27*(12), 988-993.

Troncoso, R., Diaz-Elizondo, J., Espinoza, S. P., Navarro-Marquez, M. F., Oyarzun, A. P., Riquelme, J. A., . . . Lavandero, S. (2013). Regulation of cardiac autophagy by insulin-like growth factor 1. *IUBMB Life, 65*(7), 593-601. doi: 10.1002/iub.1172

Tsoporis, J., & Leenen, F. H. (1988). Effects of arterial vasodilators on cardiac hypertrophy and sympathetic activity in rats. *Hypertension, 11*(4), 376-386.

Tsukagoshi, H., Shimizu, Y., Kawata, T., Hisada, T., Shimizu, Y., Iwamae, S., . . . Mori, M. (2001). Atrial natriuretic peptide inhibits tumor necrosis factor-alpha production by interferon-gamma-activated macrophages via suppression of p38 mitogen-activated protein kinase and nuclear factor-kappa B activation. *Regul Pept, 99*(1), 21-29.

Tsuruda, T., Kato, J., Kitamura, K., Kuwasako, K., Imamura, T., Koiwaya, Y., . . . Eto, T. (1998). Adrenomedullin: a possible autocrine or paracrine inhibitor of hypertrophy of cardiomyocytes. *Hypertension, 31*(1 Pt 2), 505-510.

Turcani, M. (2008). Biphasic dose-dependent modulation of cardiac parasympathetic activity by moxonidine, an imidazoline I1-receptor agonist. *J Cardiovasc Pharmacol, 52*(6), 524-535. doi: 10.1097/FJC.0b013e3181907146

Turner, N. A., Das, A., Warburton, P., O'Regan, D. J., Ball, S. G., & Porter, K. E. (2009). Interleukin-1alpha stimulates proinflammatory cytokine expression in human cardiac myofibroblasts. *Am J Physiol Heart Circ Physiol, 297*(3), H1117-1127. doi: 10.1152/ajpheart.00372.2009

van Heerebeek, L., Borbely, A., Niessen, H. W., Bronzwaer, J. G., van der Velden, J., Stienen, G. J., . . . Paulus, W. J. (2006). Myocardial structure and function differ in systolic and diastolic heart failure. *Circulation, 113*(16), 1966-1973. doi: 10.1161/circulationaha.105.587519

Van Kerckhoven, R., van Veghel, R., Saxena, P. R., & Schoemaker, R. G. (2004). Pharmacological therapy can increase capillary density in post-infarction remodeled rat hearts. *Cardiovasc Res, 61*(3), 620-629. doi: 10.1016/j.cardiores.2003.09.026

Van Tassell, B. W., Arena, R., Biondi-Zoccai, G., McNair Canada, J., Oddi, C., Abouzaki, N. A., . . . Abbate, A. (2014). Effects of interleukin-1 blockade with anakinra on aerobic exercise capacity in patients with heart failure and preserved ejection fraction (from the D-HART pilot study). *Am J Cardiol, 113*(2), 321-327. doi: 10.1016/j.amjcard.2013.08.047

Van Tassell, B. W., Toldo, S., Mezzaroma, E., & Abbate, A. (2013). Targeting interleukin-1 in heart disease. *Circulation, 128*(17), 1910-1923. doi: 10.1161/circulationaha.113.003199

van Wamel, A. J., Ruwhof, C., van der Valk-Kokshoom, L. E., Schrier, P. I., & van der Laarse, A. (2001). The role of angiotensin II, endothelin-1 and transforming growth factor-beta as autocrine/paracrine mediators of stretch-induced cardiomyocyte hypertrophy. *Mol Cell Biochem, 218*(1-2), 113-124.

Varga, Z. V., Giricz, Z., Liaudet, L., Hasko, G., Ferdinandy, P., & Pacher, P. (2014). Interplay of oxidative, nitrosative/nitrative stress, inflammation, cell death and autophagy in diabetic cardiomyopathy. *Biochim Biophys Acta*. doi: 10.1016/j.bbadis.2014.06.030

Vauquelin, G., De Backer, J. P., Ladure, P., & Flamez, A. (1999). Identification of I1 and I2 imidazoline receptors in striatum membranes from different species. *Ann N Y Acad Sci, 881*, 135-143.

Wan, S. H., Vogel, M. W., & Chen, H. H. (2014). Pre-Clinical Diastolic Dysfunction. *J Am Coll Cardiol, 63*(5), 407-416. doi: 10.1016/j.jacc.2013.10.063

Wang, B. W., Wu, G. J., Cheng, W. P., & Shyu, K. G. (2013). Mechanical stretch via transforming growth factor-beta1 activates microRNA-208a to regulate hypertrophy in cultured rat cardiac myocytes. *J Formos Med Assoc, 112*(10), 635-643. doi: 10.1016/j.jfma.2013.01.002

Wang, H. J., Wang, W., Cornish, K. G., Rozanski, G. J., & Zucker, I. H. (2014). Cardiac Sympathetic Afferent Denervation Attenuates Cardiac Remodeling and Improves Cardiovascular Dysfunction in Rats With Heart Failure. *Hypertension*. doi: 10.1161/hypertensionaha.114.03699

Wang, W., Zhu, W., Wang, S., Yang, D., Crow, M. T., Xiao, R. P., & Cheng, H. (2004). Sustained beta1-adrenergic stimulation modulates cardiac contractility by Ca2+/calmodulin kinase signaling pathway. *Circ Res, 95*(8), 798-806. doi: 10.1161/01.RES.0000145361.50017.aa

Ward, M. L., Crossman, D. J., & Cannell, M. B. (2011). Mechanisms of reduced contractility in an animal model of hypertensive heart failure. *Clin Exp Pharmacol Physiol, 38*(10), 711-716. doi: 10.1111/j.1440-1681.2011.05563.x

Weber, F., Brodde, O. E., Anlauf, M., & Bock, K. D. (1983). Subclassification of human beta-adrenergic receptors mediating renin release. *Clin Exp Hypertens A, 5*(2), 225-238.
Weber, K. T., Brilla, C. G., & Campbell, S. E. (1992). Regulatory mechanisms of myocardial hypertrophy and fibrosis: results of in vivo studies. *Cardiology, 81*(4-5), 266-273.
Webster, J., & Koch, H. F. (1996). Aspects of tolerability of centrally acting antihypertensive drugs. *J Cardiovasc Pharmacol, 27 Suppl 3*, S49-54.
Weeks, K. L., & McMullen, J. R. (2011). The athlete's heart vs. the failing heart: can signaling explain the two distinct outcomes? *Physiology (Bethesda), 26*(2), 97-105. doi: 10.1152/physiol.00043.2010
West, J. B. (1990). *Best and Taylor's physiological basis of medical practice*. (12e éd.).
Widlak, P., Li, P., Wang, X., & Garrard, W. T. (2000). Cleavage preferences of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease) on naked DNA and chromatin substrates. *J Biol Chem, 275*(11), 8226-8232.
Wilson, E., Mai, Q., Sudhir, K., Weiss, R. H., & Ives, H. E. (1993). Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. *J Cell Biol, 123*(3), 741-747.
Winegrad, S., & Robinson, T. F. (1978). Force generation among cells in the relaxing heart. *Eur J Cardiol, 7 Suppl*, 63-70.
Wolk, R., Kane, K. A., Cobbe, S. M., & Hicks, M. N. (1999). Facilitation of spontaneous defibrillation by moxonidine during regional ischaemia in an isolated working rabbit heart model. *Eur J Pharmacol, 367*(1), 25-32.
Woodcock, E. A. (2007). Roles of alpha1A- and alpha1B-adrenoceptors in heart: insights from studies of genetically modified mice. *Clin Exp Pharmacol Physiol, 34*(9), 884-888. doi: 10.1111/j.1440-1681.2007.04707.x
World Health Organization. (2014, 25 jun 2014). Global Health Observatory Data Repository. Number of deaths by cause. Repéré le 11 nov 2014 2014 à http://apps.who.int/gho/data/node.main.CODWORLD?lang=en
Wrigley, B. J., Lip, G. Y., & Shantsila, E. (2011). The role of monocytes and inflammation in the pathophysiology of heart failure. *Eur J Heart Fail, 13*(11), 1161-1171. doi: 10.1093/eurjhf/hfr122
Yamada, T., Nagata, K., Cheng, X. W., Obata, K., Saka, M., Miyachi, M., . . . Yokota, M. (2009). Long-term administration of nifedipine attenuates cardiac remodeling and diastolic heart failure in hypertensive rats. *Eur J Pharmacol, 615*(1-3), 163-170. doi: 10.1016/j.ejphar.2009.05.028
Yamamoto, K., Ikeda, U., & Shimada, K. (1997). Natriuretic peptides modulate nitric oxide synthesis in cytokine-stimulated cardiac myocytes. *J Mol Cell Cardiol, 29*(9), 2375-2382. doi: 10.1006/jmcc.1997.0472
Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., . . . Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature, 332*(6163), 411-415. doi: 10.1038/332411a0
Yarbrough, W. M., Baicu, C. F., Mukherjee, R., Van Laer, A. O., Rivers, W. T., McKinney, R. A., . . . Spinale, F. G. (2014). Cardiac Restricted Overexpression Or Deletion Of Tissue Inhibitor Of Matrix Metalloproteinase-4. *Am J Physiol Heart Circ Physiol*. doi: 10.1152/ajpheart.00063.2014
Yeh, C. C., Li, H., Malhotra, D., Turcato, S., Nicholas, S., Tu, R., . . . Mann, M. J. (2010). Distinctive ERK and p38 signaling in remote and infarcted myocardium during post-

MI remodeling in the mouse. *J Cell Biochem, 109*(6), 1185-1191. doi: 10.1002/jcb.22498

Yelamarty, R. V., Moore, R. L., Yu, F. T., Elensky, M., Semanchick, A. M., & Cheung, J. Y. (1992). Relaxation abnormalities in single cardiac myocytes from renovascular hypertensive rats. *Am J Physiol, 262*(4 Pt 1), C980-990.

Yndestad, A., Marshall, A. K., Hodgkinson, J. D., Tham el, L., Sugden, P. H., & Clerk, A. (2010). Modulation of interleukin signalling and gene expression in cardiac myocytes by endothelin-1. *Int J Biochem Cell Biol, 42*(2), 263-272. doi: 10.1016/j.biocel.2009.10.021

Yokoyama, T., Vaca, L., Rossen, R. D., Durante, W., Hazarika, P., & Mann, D. L. (1993). Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. *J Clin Invest, 92*(5), 2303-2312. doi: 10.1172/jci116834

Yoro Sy, G., Urosevic, D., Fellmann, L., Greney, H., Bousquet, P., & Feldman, J. (2008). G-protein inwardly rectifying potassium channels are involved in the hypotensive effect of I1-imidazoline receptor selective ligands. *J Hypertens, 26*(5), 1025-1032. doi: 10.1097/HJH.0b013e3282f5ed44

Yoshimitsu, M., Higuchi, K., Dawood, F., Rasaiah, V. I., Ayach, B., Chen, M., . . . Medin, J. A. (2006). Correction of cardiac abnormalities in fabry mice by direct intraventricular injection of a recombinant lentiviral vector that engineers expression of alpha-galactosidase A. *Circ J, 70*(11), 1503-1508.

You, H. W., Chen, X., You, H. J., Zhang, Y. Q., Cai, Y., & Liu, G. Z. (2006). [Role of endothelin-1 and its receptors on hypertrophy or proliferation of cultured cardial cells]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao, 28*(4), 520-523.

Yue, P., Massie, B. M., Simpson, P. C., & Long, C. S. (1998). Cytokine expression increases in nonmyocytes from rats with postinfarction heart failure. *Am J Physiol, 275*(1 Pt 2), H250-258.

Zacharia, J., Hillier, C., & MacDonald, A. (2004). Alpha1-adrenoceptor subtypes involved in vasoconstrictor responses to exogenous and neurally released noradrenaline in rat femoral resistance arteries. *Br J Pharmacol, 141*(6), 915-924. doi: 10.1038/sj.bjp.0705690

Zaugg, M., & Schaub, M. C. (2004). Cellular mechanisms in sympatho-modulation of the heart. *Br J Anaesth, 93*(1), 34-52. doi: 10.1093/bja/aeh159

Zhang, J., & Abdel-Rahman, A. A. (2005). Mitogen-activated protein kinase phosphorylation in the rostral ventrolateral medulla plays a key role in imidazoline (i1)-receptor-mediated hypotension. *J Pharmacol Exp Ther, 314*(3), 945-952. doi: 10.1124/jpet.105.087510

Zhang, J., & Abdel-Rahman, A. A. (2006). Nischarin as a functional imidazoline (I1) receptor. *FEBS Lett, 580*(13), 3070-3074. doi: 10.1016/j.febslet.2006.04.058

Zhang, J., & Abdel-Rahman, A. A. (2008). Inhibition of nischarin expression attenuates rilmenidine-evoked hypotension and phosphorylated extracellular signal-regulated kinase 1/2 production in the rostral ventrolateral medulla of rats. *J Pharmacol Exp Ther, 324*(1), 72-78. doi: 10.1124/jpet.107.129049

Zhang, W., Chancey, A. L., Tzeng, H. P., Zhou, Z., Lavine, K. J., Gao, F., . . . Mann, D. L. (2011). The development of myocardial fibrosis in transgenic mice with targeted overexpression of tumor necrosis factor requires mast cell-fibroblast interactions. *Circulation, 124*(19), 2106-2116. doi: 10.1161/circulationaha.111.052399

Zhao, D., Pandey, K. N., & Navar, L. G. (2010). ANP-mediated inhibition of distal nephron fractional sodium reabsorption in wild-type and mice overexpressing natriuretic peptide receptor. *Am J Physiol Renal Physiol, 298*(1), F103-108. doi: 10.1152/ajprenal.00479.2009

Zhu, Q. M., Lesnick, J. D., Jasper, J. R., MacLennan, S. J., Dillon, M. P., Eglen, R. M., & Blue, D. R., Jr. (1999). Cardiovascular effects of rilmenidine, moxonidine and clonidine in conscious wild-type and D79N alpha2A-adrenoceptor transgenic mice. *Br J Pharmacol, 126*(6), 1522-1530. doi: 10.1038/sj.bjp.0702429

Zhu, W. Z., Wang, S. Q., Chakir, K., Yang, D., Zhang, T., Brown, J. H., . . . Xiao, R. P. (2003). Linkage of beta1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca2+/calmodulin kinase II. *J Clin Invest, 111*(5), 617-625. doi: 10.1172/jci16326

Ziskind, B., & Halioua, B. (2004). La conception du coeur dans l'Egypte ancienne. *Med Sci (Paris), 20*(3), 367-373. doi: 10.1051/medsci/2004203367

Zucker, I. H., Xiao, L., & Haack, K. K. (2014). The central renin-angiotensin system and sympathetic nerve activity in chronic heart failure. *Clin Sci (Lond), 126*(10), 695-706. doi: 10.1042/cs20130294